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Proceedings from the symposium on
astric Ion Transport

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Foreword

In 1477 the first Swedish university was founded in Uppsala. Consequently the University celebrated its 500th year anniversary in 1977 This whole year was devoted to numerous symposia congresses and seminars in different fields of science

One of these symposia brought together a group of scholars from different parts of the world with a common interest in Gastric Ion Transport. During some intense sessions between July 24th and 28th the most recent progress was reported and discussed This field could for many years be characterized by a state of stagnation but in recent years a pronounced change seems to have occurred New avenues of approach have been entered due to many new technical innovations The projection of functional events on morphological substrates should also be appreciated It is an overwhelming impression that the problem of gastric acid formation and its associated transports of ions and water are more and more approaching subcellular and molecular levels Secretion physiology and membrane biophysics are slowly being linked together

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Reasons and Trends in Gastric Research

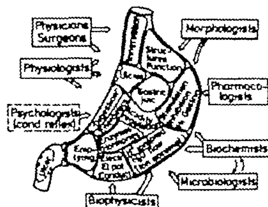
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Received 1 February 1978

Ladies and Gentlemen

As an introduction to this Symposium I have been asked to give a brief review of the development of gastric research during the last fifty years This is roughly the time-span of my own active interest in the field My story will necessarily be a personal review It will deal with the history or rather the chronology of the developments or contributions to the field from various sources The title is reasons and trends With reasons I mean the driving forces behind the research or the discoveries which led from one step to next step and how progress was promoted by the results of research workers from other sciences Of course I can neither logically nor completely pursue this theme It will be a sketch of personal recollections supported by some diagrams



The Conquest of the Stomach Country

The time before 1950

My first encounter with the field was in relation to a stomach ulcer diagnosis of a relative I was a kid and I heard of strange words like test meals stomach pumping and hypersecretion Coming to the medical school it was some 50 years ago I had to learn about their significance I cannot remember that anatomy gave much but in the histology course we were told that there were certain cells in the stomach wall called the acidophilic cells which probably produced the gastric acid and there were other cells which produced the digestion enzymes The acidophilic cells or as we call them today the oxyntic cells had got their names because they were stained by acid dyes The localisation of the acid production to these particular cells was more or less a pure guess however it turned out to be correct much later In the physiology-chemistry course we heard that the origin of the gastric acid HCl by and large was unknown It looked as if the material was taken from the blood because one stressed that digestion was accompanied by the so called alkaline tide in the urine The dominating issue of our teaching was the practical clinical one It was largely a question of how to diagnose stomach ulcer in patients Ulcers were supposed to be caused by the hyper-production of the hydrochloric acid which could corrode the stomach or the duodenal wall The terms hyperacidity and hypersecretion which had impressed me earlier came back as practical and important problems in clinical medicine In fact in the 20-30-ties the physicians main guide for treating the patient prescribe diet and so on was the determination of the so called free acidity after testmeals The clinical importance of these problems became one reason why a new generation of physiologists including myself devoted their interest and efforts to work on the mechanism of the gastric acid production and its corollary the acidity regulation Another reason was the belief that newly emerged concepts from the physical chemistry would be useful in gastric acid research New electrolyte distribution phenomena had been described by Donnan (who incidentally already in 1911 had proposed a gastric acid theory) The pH-concept and convenient methods of determining pH had come forward from about 1930 and acid base regulation studies had been introduced by van Slyke

In this context it may be emphasized how new techniques have been the starting point the reason for new lines of investigations of the

gastric juice problems I myself remember how happy I was to discover the usefulness of electrometric micro-chloride analysis to say nothing about the flamephotometer flown in from the USA right after the war Now we could start to tackle the alkali ions which was almost impossible earlier

By degrees it was realized that the production of hydrochloric acid was a case of ion accumulation The term was borrowed from the plant physiologists who had studied the potassium uptake in plants from the soil Potassium accumulation may be of the order 40:1 between cells and the environment In the same terms the hydrogen ion accumulation in the stomach is of the order of 1 million times reflecting the pH difference between pH about 7 in the plasma and the pH 1 in the gastric acid In attempts to explain the potassium accumulation in plant cells Osterhout and others introduced concepts from electrochemistry founded by Planck and Nernst Hypotheses and formulas with ion mobilities or partition coefficients became popular This particular aspect of general physiology was called ion accumulation the modern term transport processes came in much later The introduction of diffusion and electro-chemical concepts into gastric physiology is a good example of how apparently unrelated problems in d fferent branches of science can act as mutual stimulus when the research parties have become interested in one anothers work Such cross fertilisation has indeed been the reason for many developments and new trends in our gastric research

Now looking back it may be stated that the physical chemistry so proudly introduced d cades ago has failed in giving a solution to our main riddle the enormous hydrogen ion accumulation in the stomach gastric acid Of course it does not mean that physical chemistry was useless in the discuss ons of the gastric acid problems One spin-off of my physical chemical efforts when working on the cat s stomach was the realisation that the acidity regulation at least partly was due to an electrolyte interdiffusion process in the stomach what has become known as back diffusion I me ely came to understand that some kind of extra boosting was necessary This must be active in the sense that it requires expenditu e of energy derived from chemical sources

The time became ripe for the biochemists to enter the gastric research This happend oughly during the 1940-ties It was the energy rich phosphoric acid ester compounds which first came into focus and gradually

the energy releasing enzymes were transplanted to the gastric realms As you know it has taken some time for the biochemists to take over Perhaps it is justified to state that the dominating trend of today is seen in the studies of the coupling processes between ion transport and energetics There are very few papers in this Symposium which do not somehow mention ATP The first informative studies on the overall energetics were performed on isolated specimens of the gastric mucosa the technique which was introduced during and after World war II The success with the isolated superfused mucosa mounted in plastic chambers marked a very great step forward It could be stimulated with a well defined stimulant histamine and many parameters could be studied with less ambiguity then in work on stomachs in situ One early result was the demonstration that the overall effect of the H^+ ion secretion into the mucosal solution side was accompanied by a corresponding increase in the alkalinity of the nutrient solution there was a 1 1 process between H^+ and OH^- (an observation which also shed some light on the old phenomena of an alkaline tide in the urine following a meal) Well we all know what a state of refinement the chamber methods have reached in the recent years

Before I proceed with the exciting time at the end of the 1930-ties when the isotope labelling came into general use I should like to do some justice to some more old-fashioned approaches which were and still are of importance for the medical sides of the gastric problems The surgeons had added quite a great deal of knowledge about the nervous mechanisms of the gastric acid production revealed in some widely used surgical operations So had the physicians and pharmacologists made contributions on gastric juice stimulants and inhibitors In the late thirties one had a feeling that the old gastrin of Edkins from 1905 was dethroned by the powerful compound histamine By degrees the more or less unitarian arguments subsided Nowadays we acknowledge a whole series of active substances in the stomach machinery The influence of the whole body status via hormones or nervous actions has been the subject of medical psychologists From Pavlov s conditioned reflexes one has turned to the influ ence of stress as one genetic factor in stomach or duodenal ulcer formation

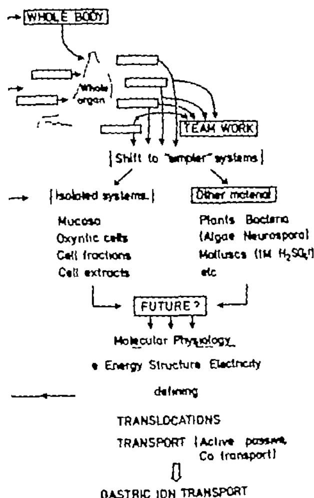
But let us return to the electrolyte transport processes This term superseded slowly the prevailing vocabulary of ion accumulation and ion permeability Perhaps this marked a realisation that the purely phe-

nomenological descriptions delivered by the experimentalists had to be supplemented by analyses in the depth of the driving forces the nature of the permeability coefficients etc In short a study of the kinetics of ion transport became pertinent A pioneer in this field was August Krogh Working in Copenhagen he had access to one of the first atomic establishments created around Niels Bohr He could use labelled ions for his research on the frog skin permeability and study the kinetics of salt uptake I think the first time I ever heard the word active transport was in a lecture by Krogh in 1937 in England Anyhow Krogh and his collaborator Ussing initiated a real flood of transport studies on all sorts of living membranes and also model membranes These studies were slowed down by the war but around 1950 isotopic investigations were also performed in stomach physiology

The years after 1950.

In the beginning of the 1950-ties I more or less left the stomach field Karl Johan Öbrink had now introduced the isotope techniques in our laboratory and had already started his own important gastric research He had got his own pupils some of them who will now help him to carry these Symposia days I believe I have at least partially explained some reasons why we are assembled here in Uppsala this week In fact I have felt that it ought to have been KJ Öbrink rather than myself who should tell the story about the recent decades of stomach work On the other hand perhaps a by-stande could more easily appreciate the overall direction in which a esearch field developes and perhaps be able to read the trends which lead to the future

The precise locations of the present research lines are difficult to perceive One clear trend however is that one is moving from the whole body and the whole organ approach and there is a definite shift to simpler systems I may refer you to the adjacent diagram. Due to the fact that so many different scientific specialities have gone into gastric research it has become increasingly difficult for a single person or even a small group to follow up the project This circumstance together with the need of specialists and sophisticated instrumentation has made team-work necessary Physiologists microbiologists and electronic engineers may be regular members of a research team of these modern times This trend has many advantages On the other hand one cannot escape the feeling



From Here to the Future

that there will be a widening of the gap between the medical side the physicians and surgeons who have to deal with the pathological disturbances of our stomachs and those who do so called basic research on the same object Now when much of the stomach conquest has been directed towards isolated systems one must not forget the initial human and medical reasons for gastric research The ulcus problem is still unsolved and the ulcus symptoms may remain distressing conditions in our stomachs We have to admit however that modern pharmacology has created powerful agents for some control of the acid secretion more than often only symptomatical I believe that there is a long way to go before we get a full understanding how the stomach acts in its integrated state in the whole body

The work on isolated systems which began with pieces of frog mucosa have now advanced to isolated cell structures and even substructures of cells A great deal of the transactions of this symposium will certainly deal with such investigations There is another trend which perhaps has not been appreciated fully and that is that a great deal of the key-problems of acid formation are not confined to the higher animals In fact mechanisms for pumping hydrogen ions so called proton pumps exist in plant metabolism and in bacterias As regards the electrical phenomena a great deal can be learnt from work on plant cells particularly algae as Nitella Ion transport and electrical phenomena have been studied by plant physiologists with remarkable success The salivary gland of a mollusc Dolium galea which lives in the Mediterranean is a very interesting object This produces sulphuric acid is about 1 molar in other words about 10 times stronger than the mammalian hydrochloric acidity!

Looking towards the future

From a reading of the present trends I feel that the future of gastric research will be a part of something which might be called molecular physiology This may be a provisional term for a broad and deep research which embarks on the interrelations between energy structures and forces It becomes increasingly more evident that one cannot separate transport processes from the structures and that the structures in turn are functions of the driving forces The invisible forces and the visible structures are mutually dependent on one another and are inseparable! The problems in the future will not only be concerned with the transport forces for ions and molecules they must also include analyses of the translocation forces of larger structures like the vesicles To distinguish between translocation of structures and transport of dissolved material may be a difficult task The same statement may apply to the discrimination between active and passive transport forms In fact neither exists entirely separated from the other form Again it is a question of coupling to energy Everything on the molecular level is coupled processes Even the transport of water so important in the gastric juice production will certainly be disclosed as a form of co-transport

In the march towards the future many obstacles have to be overcome not the least proper problem definitions Many theories are not yet

born and maybe there will be some hope that the modern non-linear irreversible thermodynamics will supply guides for the understanding of structure formation Without organized structures there will be no membranes and without membranes life would not exist!

The future will certainly be a challenging and exciting time for the next generations of gastric research workers –

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Effects of Chronic Pentagastrin Stimulation on Parietal Cell Size in Rat Gastric Mucosa

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ABSTRACT Pentagastrin was injected in rats every eight hours for two weeks Quantitative electron microscopical studies of the gastric mucosae revealed parietal cell hypertrophy in these rats There was also an increase in the proportion of mucosa occupied by the parietal cells and by the epithelial cells respectively

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The effects of chronic pentagastrin stimulation on the gastric mucosa have previously been studied by several investigators A trophic effect can be demonstrated in normal rat oxyntic mucosa resulting in an increased synthesis of DNA and RNA (1 2) Furthermore it has been reported that there is an increase in the height of the mucosa (3 4) and an hyperplasia of the parietal cells (3 5)

In the present study the gastric mucosa from pentagastrin stimulated rats was studied with quantitative electron microscopical methods in order to analyze any changes which might occur in the size and number of the parietal cells

Material and Methods

The study was performed on 16 male Sprague Dawley rats which were 3 1/2 months old when they were killed Eight of the animals were given an injection of pentagastrin (250 μg/kg body weight) every eight hours for 14 days The pentagastrin was dissolved in 0 05 molar NH_4HCO_3 (pH 8 2) in physiological saline and given in Calcitonin Diluent B (15% hydrolyzed gelatin prepared by Armour Pharmaceutical Co) 1 ml of this mixture was injected each time (6 7) The remaining eight rats served as

controls and received no injections Immediately before sacrifice the rats in the control group weighed 410 ± 13 g (mean ± S D n=8) and those in the pentagastrin group 388 ± 13 g

Before sacrifice the rats were fasted for 24 hours in individual wire-mesh bottom cages with free access to drinking water The last injection of pentagastrin was given about 11 hours before the animals were killed To avoid any diurnal variations all rats were killed between 9 a m and noon and in order to minimize the differences in the fixation four control rats and four stimulated rats were sacrified on two consecutive days with the fixation fluid made up once daily The rats were anaesthetized with an intraperitoneal injection of sodium pentabarbitol (0 03 mg per g of body weight) The abdomen was opened and a cannula was inserted into the abdominal aorta in a retrograd direction just above the bifurcation A hole was then cut into the portal vein and infusion of rinsing fluid was started through the cannula The rinsing fluid was a slightly modified Tyrode solution (for composition see ref 8) containing heparin and procain and it was delivered by means of a peristaltic pump at a rate of about 30 ml/min By tying off the mesenterial vessels and by clamping the aorta just above the diaphragm most of the fluid was directed to the upper abdominal organs

After about a minute the blood vessels on the gastric wall appeared empty of blood and the rinsing fluid was substituted by fixation fluid This was identical to the rinsing fluid but contained in addition 3% formaldehyde 4% glutaraldehyde and 0 05% picric acid (9) (pH 7 2 1 500 mosmol) Simultaneously a few milliliters of the fixative was injected into the gastric cavity The perfusion of fixative continued for 10 minutes at the same rate (30 ml/min) after which a portion of the gastric wall was excised from the greater curvature about 5 mm distal to the borderline of the forestomach This tissue was cut into small pieces and was kept in the fixative at 4°C for another 3 hours It was then rinsed in Tyrode postfixed in 1% OsO_4 in Tyrode at 4°C for 2 hours dehydrated in rising concentrations of ethanol and embedded in epon Ultramicrotome sections which covered the entire thickness of the mucosa were cut roughly perpendicular to the mucosal surface the thickness of the sections was estimated to 80 nm After contrasting with lead hydroxide and uranyl acetate the sections were examined in a Philips EM 300 electron microscope

In survey electron micrographs trapezoid areas were marked which were limited by the surface of the mucosa the bottom of the glands and two parallel lines about 50 to 200 µm apart Within these areas all parietal cells were photographed at primary magnifications of about 1 200 times In the control group 383 parietal cells were photographed vs 350 in the stimulated group In order to avoid identification problems only such parietal cells were photographed which exhibited the nucleus in the section Using Weibel s multipurpose grid (10) the area was then determined for each parietal cell profile

On the survey electron micrographs a similar grid was used to obtain the volume densities of the epithelial cells and of the parietal cells in reference to the mucosal volume (i e the percentage of mucosal volume muscularis mucosae not included which was occupied by the epithelial cells and by the parietal cells respectively)

Finally the thickness of the mucosa (excluding the muscularis mucosae) was measured in one micron thick survey sections

The results are given as mean values ± S D (n=8)

Results

There were no obvious differences between the two groups of rats with respect to the ultrastructure of the gastric mucosa In particular the shape and the subcellular morphology of the parietal cells appeared quite similar in the two groups

The thickness of the mucosa was 0 65 ± 0 10 mm in the control rats and 0 65 ± 0 08 mm in the stimulated rats The mean profile area of the parietal cells was 89 ± 7 μm^2 in the control rats vs 104 ± 9 μm^2 in the stimulated rats The difference is statistically significant (p<0 005) and corresponds to a ~25% larger cell volume in the stimulated rats Permitting the assumption that the parietal cells are spheres (they are often ellipsoidal 11) and that they were all sectioned through their centers (the nucleus is in the central part of the cells) then we can obtain a rough estimate of their mean volumes In the control rats the volume was thus calculated to 635 ± 76 μm^3 and in the pentagastrin treated rats 797 ± 111 μm^3

The volume density of the epithelial cells was 44 ± 4 5% in the control rats and 52 ± 4 9% in the stimulated rats the difference is statistically significant (p<0 005)

The volume density of the parietal cells was 12 ±1 5% in the control rats and 16 ± 2 7% in the stimulated rats also this difference is statistically significant (p<0 005)

By dividing the parietal cell volume densities by the figures for mean parietal cell volumes we can approximate the number of parietal cells per unit volume of mucosa In the control rats this number averaged 194 000 ± 24 000 per mm^3 and in the stimulated rats 198 000 ± 32 000

Discussion

The estimated mean parietal cell volume in normal rats is ~40% smaller than that in a previous study (8) Moreover figures for the parietal cell volume density and epithelial cell volume density are 15 20% lower The estimated number of parietal cells is ~35% higher These variances are probably due to the use of two different fixatives in the previous investigation 2% glutaraldehyde (470 milliosmoles) was employed

The data show that the trophic effects of chronic pentagastrin stimu lation in rats also include an hypertrophy of the parietal cells thi leads to an increase of their mean volume by ~25% Theoretically this could also be due to the solvent but this does not seem very likely Neither the hyperplasia of the parietal cells nor the increase in mucosal thickness reported by previous investigators (3-5) could be confirmed However the proportion of epithelial cells rose from 44 to 52% Differences in the preparation of the tissue as well as their use of light microscopic morphometry may well explain the divergence in results

The growth of the parietal cells whether by hyperplasia or by hyper- trophy is well correlated to alterations in the acid secretory capa city Thus Stanley et al (5) who gave the same doses of pentagastrin to rats obtained an 80% increase in both basal and maximal acid output With injections twice daily of 2 mg pentagastrin for three weeks Crean et al (3) demonstrated a four-fold increase in acid output It is conceiv able that longer periods of treatment with pentagastrin as well as higher doses would accentuate the morphological changes in the gastric mucosa

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Ultrastructural Changes in Mouse Parietal Cells after High H^+ Secretion

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ABSTRACT: Further elucidation of parietal cell ultrastructural changes found during diff rent secretory stages particularly when acid secr tion eases r described Perfused stomachs of anaesthesized mice were monitored f r acid secr tion and fixed at known periods f secretory activity and examined by lectron microscopy The characteristic morphology f tive and non-secreting parietal cells was confirmed and some intermediate tages wer observed The long tanding interpretation that tubulovesicular membranes become continuous with the plasma membrane could not be confirmed Alternative possible processes that may be involved include 1 the closing off and internalization of the intracellular canaliculi; 2 f rmation of vacuoles and multivesicular bodies; 3 p esence of endocytotic vesicles and coated vesi les; 4 and the appearance f concentri membran profiles flattened vesicle and pentalaminar bodies It is suggested that these morphological features and other processes which may not be apparent by current ti sue preparation techniques are involved in the movement f membrane to and from the tubulovesicular compartment and the plasma membrane

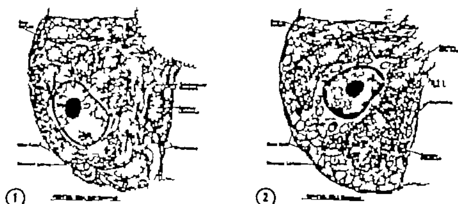
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Based on confirmatory evidence from various laboratories there is general agreement on the ultrastructural appearanc of the a tively secreting oxyntic r pari tal cell and its non-secreting configuration (1 10) The mo t triki g morphological feature f the non-secreting parietal cell is the abundance of tubulovesicular membrane whereas microvilli are few and sparse On the other hand stimulated secreting cells ar characterized by a marked decrea in the tubulovesicular sy tem and an increase in the luminal plasma membrane with numerous longated microvilli Upon return to the non-secretory state the pari tal cell tructure reve ts to the inactive configuration It has been uggested that the cytoplasmic tubules or vesicles contribute directly

towards the increased cell surfa e by direct fusion (11-13) The reduction in the surface plasma membrane has been interpreted to be mediated by endocytosis by internalization of microvilli (4) or by formation of pentalaminar membrane structures (4 7) Although it seems probable that all these processes are involved the question remains as to whether they represent the sole or the major mechanism responsible for the transition from the active to the inactive state In this presentation we would like to consider some ultrastructural bservations on parietal cells from stomachs of mice fixed at known periods of s cretory activity In addition observations on parietal cells from cat stomachs harboring gastric spirilla ar included

Young adult male mice fasted overnight and ana sthesized with sodium pentobarbitone were prepared for gastric perfusion as previously described (9) Stomachs were perfused and the collected solution was monitored for acid secretion at 15 min intervals Unstimulated control mice as well as mice stimulated by injection of histamine (0 23 mg free base/ml/h), followed by addition of carbachol (0 5 mg/ml) in the gastric perfusate were used At known levels of secretory activity the stomachs were fixed f r electron microscopy by substituting the gastric perfusate with a solution containing formaldehyde glutaraldehyde and trinitrocresol Tissu s were embedded in poxy resin after osmication and uranyl acetate en bloc treatment The specimens of cat stomach were fixed in buffered OsO_4 and treated with uranyl acetat prior to embedment Thin sections were examined after staining with uranyl acetate and lead citrate

The structure f the mammalian parietal cell as related t gastric H^+ ion secretion has been well defined in recent ye rs nd the salient features as determined by electron microscopy are illustrated in the drawings shown in Figs 1 nd 2 The non-s cr ting cell configuration was charact ristic of parietal cells in mice secreting usually no more than 0 03 ueq/g body wt/15 min for several hours Thes cells are invariably packed with tubul s and vesicl s which occupy 30 to 38% of the cell volume while the st bby mi rovilli compris only 3 to 9% of the cell volume (9) The other c llular components remain r latively unchanged in different se ret ry states Mito hondria in pari tal c lls are large and abundant and oc upy about 22 to 34% of the cytoplasm In



some non-secreting pari tal cells multivesi ular bodies and lysosomes re particularly prominent

In contrast to the inactive pari tal cell experimentally timulated pari tal cells from mice secreting as much as 1 ueq/g body wt/15 min at th peak period of secretion have the ppea ance illustr ted in Fig 2 In these ells the tubul vesicula compartment has been greatly virtually depl ted nd the i a great increas in the numbe d length f microvilli which tend t occlude the luminal space f the canaliculi In mi timulated with insulin (9) which produc only mod rately high rates f acid secretion the mi rovillar volume increases to about 21% of the t tal ell volume whil the tubulovesicular system is reduced to 3 t 5%

It must be tressed that the experimentally produced extreme functional tates illustrated in the drawings are not found as the predominant pari tal cell configuration in amples from fasted refed mice Normally pari tal cells exhibit some int rmediate morphology which probably represents low r intermediate levels f ecretory ctivity

A recently eported bservation (14) which may be important in the present consideration f memb ane transposition between mi rovilli and tubulovesicular membrane is the int rnalization f the intracellular canaliculi As illustr ted in Fig 3 the pari tal ell from non-secreting control mouse contains eve al di tended canaliculi with a few short tubby microvilli The content f these canaliculi is clear in marked contrast with the adj cent lumen f th gastric gland which is

the energy releasing enzymes were transplanted to the gastric realms As you know it has taken some time for the biochemists to take over Perhaps it is justified to state that the dominating trend of today is seen in the studies of the coupling processes between ion transport and energetics There are very few papers in this Symposium which do not somehow mention ATP The first informative studies on the overall energetics were performed on isolated specimens of the gastric mucosa the technique which was introduced during and after World war II The success with the isolated superfused mucosa mounted in plastic chambers marked a very great step forward It could be stimulated with a well defined stimulant histamine and many parameters could be studied with less ambiguity then in work on stomachs in situ One early result was the demonstration that the overall effect of the H^+ ion secretion into the mucosal solution side was accompanied by a corresponding increase in the alkalinity of the nutrient solution there was a 1:1 process between H^+ and OH^- (an observation which also shed some light on the old phenomena of an alkaline tide in the urine following a meal) Well we all know what a state of refinement the chamber methods have reached in the recent years

Before I proceed with the exciting time at the end of the 1930-ties when the isotope labelling came into general use I should like to do some justice to some more old-fashioned approaches which were and still are of importance for the medical sides of the gastric problems The surgeons had added quite a great deal of knowledge about the nervous mechanisms of the gastric acid production revealed in some widely used surgical operations So had the physicians and pharmacologists made contributions on gastric juice stimulants and inhibitors In the late thirties one had a feeling that the old gastrin of Edkins from 1905 was dethroned by the powerful compound histamine By degrees the more or less unitarian arguments subsided Nowadays we acknowledge a whole series of active substances in the stomach machinery The influence of the whole body status via hormones or nervous actions has been the subject of medical psychologists From Pavlov s conditioned reflexes one has turned to the influence of stress as one genetic factor in stomach or duodenal ulcer formation

But let us return to the electrolyte transport processes This term superseeded slowly the prevailing vocabulary of ion accumulation and ion permeability Perhaps this marked a realisation that the purely phe-





8 Part of an intracellular canaliculus f a pari tal cell from a whi h had secreted 10 9 ueq/15 min but fixed when the rat had ped to 2 65 ueq/15 min Note the pleomorphic hape f the microvilli in some mi rovilli (rrows) tubula profiles re evident within the s

9 Pa t f a mouse parietal cell aft r secretion had diminished t ueq/15 min from high f 16 0 Note the presenc f a multivesicular body (MVB) well as concentri membrane profiles (CMP) flattened vesicles (FV) and coated ve icl (CV)

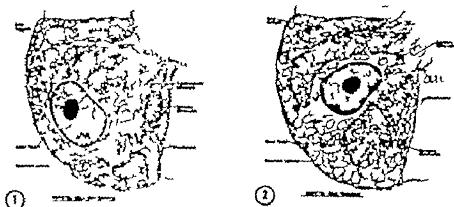
continuity between thes two membrane compartments Numerous small multivesicular bodi as well lysosomes are apparent

During the pe iod when pari tal cells are returning to an ina tive tate the microvilli seem t loose some f thei rigidity and are ften pleomorphic bulbous in shape In some f the microvilli tubular memb anes ar invaginated into the microvilla core Se rrows in Fig 8 These images r not found in actively ecr ting parietal cells but restricted to non-secreting elements tho cells r turning to the

towards the increased cell surface by direct fusion (11-13) Th reduction in the surface plasma membrane has been interpreted to be mediated by endocytosis by internalisation of microvilli (4) or by formation of pentalaminar membran structures (4 7) Although it seems probable that all these processes are involved the question remains as to whether they r present th sole or the major mechanism responsible for the transition from the a tive to the inactive state In this presentation we would like to consid r some ultrastru tural observations on parietal cells from stomachs of mice fixed at known p riods f secretory ctivity In addition observations on parietal cells from cat stomachs harboring gastric spirilla are included

Young adult male mice fasted overnight and ane sthesized with sodium pentobarbitone were prepared for gastri perfusion as previously described (9) Stomach wer perfused nd the collected solution wa monitored f r acid s cretion at 15 min interval Unstimulated control mice as well as mic stimulated by injection of hi tamine (0 23 mg free base/ml/h), followed by ddition of carbachol (0 5 ng/ml) in the gastric perfusate were used At known levels of secretory activity the stomachs we fixed for electron mi copy by substituting the gastric pe fusate with a solution containing formaldehyde glutaraldehyde and trinitrocresol Tissues were embedded in epoxy resin fter smication and uranyl acetate _en bloc_ treatme t Th specimens of cat tomach were fixed in buff red OsO_4 and treated with uranyl a etate pri to embedment Thin secti ns wer exami ed fter staining with uranyl acetate and lead citrate

The structure of the mammalian parietal cell as related to gastric H^+ ion secretion has been well d fined in recent years and the salient features as d termined by electron mi roscopy are illustrated in the drawings shown in Figs 1 and 2 The non- ecreting ell configuration was characteristic of pari tal cells in mice secr ting usually no more than 0 03 ueq/g body wt/15 min f r several hours These cells are invariably packed with tubules nd vesi les which occupy 30 to 38% of the c ll volume while the st bby microvilli ompris only 3 to 9% of the cell volume (9) The ther llular compon nts remain elatively unchanged in different e retory tat s Mit chondria in parietal cells are large and abundant and occupy about 22 to 34% of the cytoplasm In



some non-secr ting parietal cells multivesicular bodie and lysosomes re particularly prominent

In contr t to the ina tive pari tal c ll experimentally timulated parietal cell from mice ecr ting as much as 1 ueq/g body wt/15 min at the peak period of ecr tion have the appearanc illustrated in Fig 2 In these cells the tubulovesicular compartment has been gre tly or virtually depleted and ther is a great increas in the number and length of mi rovilli which tend to occlude the luminal space f the canali uli In mi timulated with insulin (9) which produce only mod rately high tes f cid se r tion the microvillar volume increases t about 21% of the t tal cell volume whil the tubulovesicula sy tem is reduced to 3 to 5%

It must be tressed that the experimentally produced extreme func tional tates illust ted in the drawings ar not found the predominant parietal cell configuration in samples from fasted or fed d mice Normally p rietal cells exhibit some intermediate morphology which probably r present low or int rmediat levels f secretory activity

A recently eported bservation (14) which may be important in the p esent consideration f membrane transposition between microvilli and tubulove ic la membrane is the internalization of the intracellular canaliculi As illust ated in Fig 3 the pari tal cell from non-se ting contr l mous contains several distended canaliculi with a few abo t tubby mic ovilli The content of these canaliculi is clear in ma ked ont ast with the adj cent lumen of the gastric gland which is

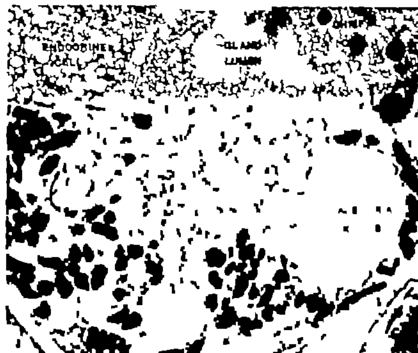
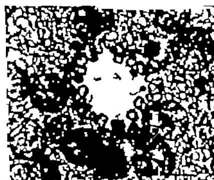


Fig 3 A low power electron micrograph f a non-se reting mouse parietal cell Note that the lumen of the gland is filled with a dens secretory product (pepsinogen) while the intracellular canaliculus appears empty An endocrine cell at upper left and chief cells t upper right

filled with a dense substance resembling pepsinogen from neighboring chief cells If the intracellular canaliculi were connected with the lumen the dense secretory product should have diffused into the canali culi Its absence therefore trongly suggests that lumen and intracellular canaliculi are separate compartments

Further evidence for the internalization of the intrac llular canaliculi is illustrated by a series of micrographs from cat parietal cells (Fig 4–6) Many mammalian stomachs harbor numerous large gastric spirilla They not only tolerate the acid environment and strong proteolytic activity of gastric secretions but require this unusual environment for their maintenance and proliferation In many parietal cells







Figs 4–6 Portions of cat parietal cell Fig 4 is a ection of an intracellular canaliculus with open lumen Fig 5 illustrates canaliculus containing a section f a gastri spirillum Fig 6 is a low power microgr ph f parietal cell containing spirilla contained within lysosomal bodies and und rgoing degeneration

from animals infected with these microorganisms the bact ria may be found p trating into the lumen f the intracellula canaliculi Furthermore these pari tal cells often contain number f dense lysosomal bodie In some f the lysosome ingl spirillum ggregates f several ba teria enclosed by the limiting membrane f the digestive va uol and ppea in various tage f tolysis One obvi us interpretation i that during acid ecretory tivity the ga tric spirilla swim down into the depths of the intracellular channels, Why they favo this presumed it f H^+ ion ecretion where the pH is 1 0 r lower is int - esting but unresolved In any vent they represent convenient ultra- tructural trace and their intracellular location suggests that during active secretion the canali ulus opens into the gland lumen Subsequently when secretion ceases and th canaliculus no longer communicates with the gland lumen the f reign intruders are trapped within the parietal ell and ultimately digested Obviously an alternative interpretation may be

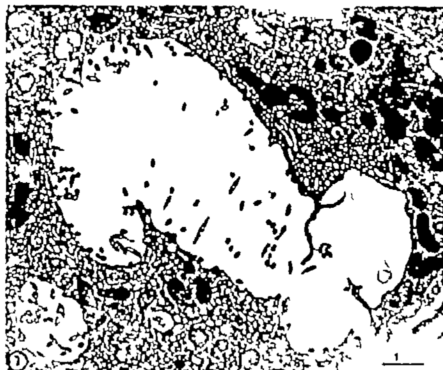


Fig 7 An electron micrograph of a mouse parietal cell returning to the ina tive state The intracellular canaliculi i bordered by a few microvilli and the cytoplasm contains abundant tubulovesicular elements

that spirilla were simply phagocytosed after they ent red the canaliculus However this seems less likely since the amount of canalicular area it self is greatly reduced in the nonsecrating parietal cell

The remaining electron micrographs (Figs 7 12) illustrate some of the structural features which are found in mouse parietal cells during recovery from high secretory activity when acid secretion was declining towards r just reached control levels In Fig 7 a lat stage in tubulovesicular reconstitution by the p rietal cell at the expense of the plasma membrane of the microvilli of the intrac llular canaliculus i illustrated The lumen of the canaliculus appears distended Although elements of the tubulovesicular system come into close approximation with the plasma membrane there is no evidence that ther is any direct



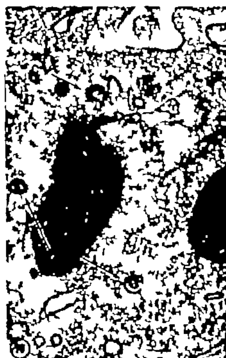


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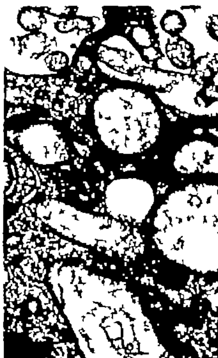


Fig 10 Sampl from same animal as Fig 9 An internalized coated vesicle (CV) as well as concentric membrane profiles (CMP) and flattened vesicles ar present but tubulovesicular elements ar not yet formed

Fig 11 A mouse parietal cell from an animal which was secreting at a rate of 20 5 ueq/15 min and ubsequently dropped to 2 5 In some cells close packed membrane profiles resembling the pentalaminar tructures ar seen

inactive state

An ubiquitous cytoplasmic component of pari tal cells during the phase of recovery from high secr tory activity is represented by the concentric membrane profiles (CMP) illustrated in Figs 8 9 and 10 These profiles have an out r diamet f 80–100 nm and cont in an inner vesicl about 40–50 nm in diameter In some sections the inner element appears to r present an infolding of the outer membrane and thus the tructure can be interpreted a cup-shaped ci tern A favorabl section in Fig 10 suggests this possibility Whether all of these concentric membrane



Fig 12 A parietal cell from animal secreting 10 meq/15 min some 45 min after a peak response f 15 meq/15 min Numerous tubulovesicular elements with enclosed vesicles are p esent Also note the lysosomal bodies

profiles result from infolding of a vesicle or actually represent a vesicle enclosed by another vesicle is not known Along with concentric membrane profiles there are impl flattened vesicles about 20-40 nm in their narrow dimension The precise origin and eventual fate f these vesicular elements is not lear but many early forming tubulovesicular membranes encl sed small vesicles (Fig 12) Another structure possibly related to the concentric profiles ar the coated vesicles still joined to the plasma memb ane (Fig 9) or free in the cytoplasm (Fig 10) Microfilaments in the microvilli and in the cytoplasm re consistently present and they have been shown (15) to be actin-like A few microtubules e also present and togethe with the filaments have been suggested as pos ibl means of internalizing plasma membrane (7)

In some cells the cytoplasm contain clear rounded or longated bodies rrounded by two l yers of l sely apposed memb anes (Fig 11) These struct re have some resemblance to the five layered membranes described in dog pa ietal cells (4) or the pentalaminar structures of the pigl t pari tal cell (7) I the mouse mat rial these membranes e not as lo ly packed and a clear spac f at least 2 nm separates the

adjacent membranes Multivesicular bodies (Figs 7 9 12) are often prominent in the non-secreting parietal cell

Observations on parietal cells of known functional activity reveal interesting structural correlates to cell activity The further understanding of just how this cell manages to change its morphology in response to stimulation will be important in learning how the stomach secretes acid Furthermore study of this rather unique cell may well add to our basic knowledge of how cells and their membranes are involved in ion secretion In parietal cells it seems reasonable to look for a morphological basis for the transposition of tubulovesicular membrane components to and from the plasma membrane as a correlate to acid secretion Although there may be segments of the process that may not be demonstrable by current microscopic techniques it seems worthwhile to explore further the structural correlates of gastric ion secretion

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The Cytoprotective Effect of Mucosal Blood Flow in Experimental Erosive Gastritis

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ABSTRACT: Blood flow through the gastric secretory epithelium of the dog was studied by radioactive microspheres under a variety of conditions that lead to enhanced H^+ for Na^+ exchange and acute erosive gastritis Aspirin in the dose range studied uniformly lead to erosions in normal or low flow states Increased flow prevented lesion formation Substances such as PCMBS and Carbenoxolone that are associated with a high rate of back-diffusion of H^+ and Na^+ efflux do not produce erosive gastritis as expected because of a large increase in mucosal perfusion Thus mucosal blood flow protects gastric mucosa from chemical injury

Gastric secretory epithelium of most mammals (1 3) including dog (4-7) presents a relatively impermeable barrier to hydrogen ions This is a fortunate characteristic that allows the development of a high level of intragastric acid ity without injury to the mucosal surface Chemical disruption of this cation barrier by a variety of substances leads to a substantial increase in H^+ back diffusion and Na^+ outflow (8) These events are usually accompanied by acute gastric mucosal lesions in the form of erosive gastritis (9)

The mechanisms of injury which contribute to or follow breaking of the cation barrier are poorly understood It is the purpose of this paper to review work done in our laboratories on the role of mucosal blood flow in this process The results of these studies suggest that blood flow may play an important role in protecting the mucosal surface from injury during high rates of H^+

pr t on Supernormal levels of blood flow also provide immunity from the
h al barrier breaker aspirin

EXPERIMENTAL DESIGN

nt f greater curve of dog stomach was housed in a lucite chamber
t al surface uppermost and blood supply intact (10) The mucosal
18 cm^2 in single chamber experiments and 14 cm^2 in those in
umened chamber was employed All studies were carried out
mals anesthetized with sodium pentobarbital

r ment the mucosal surface was covered with a known
hydrochloric acid (0 15 N) The instillate was drained by
utes and then replaced by fresh solution Net fluid ex
ned by difference in weight between fluid instilled and
n ion content was ascertained by potentiometric titration
oc m n entration by flame photometry Back-diffusion of hydrogen ions was calculated by subtracting the mass of hydrogen ions recovered from that instilled Sodium efflux was identified by the rate of appearance of Na^+ in the acid instillates

Total blood flow through the gastric segment was measured by a venous outflow technique (11) Mucosal blood flow was estimated by the method of aminopyrine clearance and/or the distribution of radioactive microspheres (12 13) All of these methods have had extensive usage in our laboratory and when used together provide a reliable way to quantitate vascular events

The mucosal surface was observed during the experiments and also examined by light and transmission electron microscopy Standard methods were

employed for fixation and staining details of which are provided in the referenced papers (16) Since sampling is such an important part of this phase of the work the following guidelines were established early in our studies and strictly adhered to Firstly in most studies biopsies were taken from one mucosal surface while analytical measurements were made simultaneously from the other in the double chamber model under precisely similar experimental conditions Secondly whenever possible the morphologist member of the team (CZ) carried out blind studies of control and test mucosa

A variety of substances known to be injurious to the mucosal epithelium were used in these experiments as stressors or "barrier breakers Aspirin bile salts and alcohol were employed because of their reliability and popularity It should be pointed out that they produce quite different lesions to the naked as well as the aided eye Alcohol injury (25% w/v) is mild with only minimal disturbance in appearance of the mucosal surface Bile salts (20 to 40 mM sodium taurocholate in 14 N HCl) produce a severe injury characterized by an eschar formation over the rugal crest Aspirin (20 mM in 0 14 N HCl) induces multiple small well circumscribed focal lesions which are similar to what is seen grossly when the mucosal surface of patients with erosive gastritis undergo endoscopic examination The lesions which follow exposure of the mucosa to p-Chloromercuribenzene Sulfonate in the concentrations employed (10 mM PCMBS in 0 1 M Tris buffer adjusted to pH 7 8 in HCl and made isosmotic with mannitol) could only be appreciated by light microscopy Bathing the mucosa with 0 25% carbenoxolone sodium dissolved in normal saline by adjusting the pH to 7 5 with Na_2CO_3 was accompanied by no apparent gross changes in the mucosa and only a mild disturbance in the rate of cell extrusion

or mucus release identified at low level electron microscopy Details of these findings will be discussed below

BLOOD FLOW AND CATION EXCHANGE IN RESPONSE TO COMMON STRESSORS

The effects of exposing gastric epithelium to weak acids has been well characterized during the years since the initial observation of Teorell that acetic acid and other barrier breakers cause a rapid disappearance of instilled acid from the stomach of the cat with a comparable gain of sodium entry into the gastric lumen (1) Similar events have been documented in pouches of canine gastric secretory mucosa and in the intact stomach of man (16) The role of blood flow in this process remained controversial until Archibald and Simons validated the technique of distribution of radioactive microspheres for measurement of gastric mucosal blood flow (12 13) Cheung and his colleagues have used this technology to document the changes in blood flow which occur during exposure of the mucosa to alcohol aspirin and bile salts (14) Hydrogen ion back-diffusion and blood flow increase in each instance The results can roughly be summarized by stating that a several fold increase in H^+ back diffusion was accompanied by only a two to three-fold increase in mucosal blood flow Aminopyrine clearance measured only 50% of the volume of mucosal blood flow indicated by microsphere distribution

MUCOSAL BLOOD FLOW DURING MILD INJURY

Para-Chloromercuribenzene Sulfonate a sulfhydryl reagent which is known to disrupt biological membranes was employed as an agent which would produce only mild injury to the mucosa during rapid rates of H^+ back diffusion (15) The relationship between H^+ back diffusion and Na^+ efflux was distinctly at unity We have observed a similar relationship for most barrier

breakers and have adopted the notion that one for one exchange of Na^+ for H^+ must have some biological significance While there is considerable variability between dogs in any group of experiments the increase in H^+ loss through the mucosa is always accompanied by an increment in sodium movement into the lumen Whether this increase in Na^+ efflux is a consequence of increased filtration and passive movement of sodium between cells or active transport through cells is not known The degree of injury usually seen at the ultrastructural level with PCMBS in neutral solution is mild characterized by loss of surface cell microvilli widening of intercellular spaces and vacuolization of mitochondria (16) There are no detectable gross mucosal changes under these conditions Exposure to PCMBS in isosmotic acid however produces an elaboration of a thick white coat of mucus over the mucosal surface The extent of histologic injury is also more severe

The relationship between H^+ back-diffusion and mucosal blood flow (as measured by microspheres) was studied in a separate group of experiments employing canine gastric segments housed in a double chamber The ratio between mucosal blood flow (MBF) and H^+ back-diffusion equalled 0 54 $\pm$ 0 13 prior to PCMBS and 0 33 $\pm$ 0 11 after PCMBS when H^+ loss increased from 1 78 $\pm$ 0 63 to 17 8 $\pm$ 2 17 μEq/min (n=4) No change in the mucosal surface was observed When the ratio of MBF to H^+ loss was reduced to 07 $\pm$ 01 by blood withdrawal massive disruption of the mucosal surface ensued These experiments clearly established a critical relationship between level of mucosal perfusion and rate of H^+ back-diffusion The high levels of mucosal blood flow appeared to protect the epithelium from PCMBS injury; a restriction of mucosal perfusion in the presence of PCMBS led to severe erosiv gastritis

CONDITIONS UNDER WHICH THE MUCOSA CAN TOLERATE A HIGH RATE OF H^+ PERMEATION

It was quite by chance that we learned that the mucosa can tolerate a high rate of H^+ back diffusion without developing erosive lesions following exposure not only to PCMBS but also to the triterpenoid cogener of licorice Carbenoxolone (17) Exposure of the mucosal surface to 0 25% carbenoxolone in an isosmotic solution of HCl led to a nearly four–fold increase in H^+ to Na^+ exchange and a comparable large increase in mucosal perfusion The major histologic finding was the in situ degeneration of mucus cells accompanied by the intracellular solubilization of mucus These experiments further support the idea that enhanced mucosal perfusion during rapid rates of H^+ can protect the mucosal surface from injury Results with PCMBS also provided a clue that such would be the case and established that reducing blood flow in the presence of a tolerable level of enhanced back diffusion would uniformly lead to injury We have not carried out this latter experiment with carbenoxolone The work of Hamza and Den Besten and Ritchie had clearly established this latter point previously in dogs as well as in monkeys (18 19) Cheung and Chang have subsequently defined the quantitative relationships under which this might occur (20) It is now quite clear that a decrease in mucosal perfusion in the presence of disruption of the mucosal barrier to cation diffusion will lead to acute mucosal lesions

PREVENTION OF ASPIRIN EROSIVE GASTRITIS BY ENHANCED MUCOSAL BLOOD FLOW

A critical question is whether increasing blood flow to the mucosa can prevent injury following exposure to barrier disruption Dr McGreevy has answered this question in the affirmative by increasing mucosal blood flow

several fold with the direct intraarterial administration of isoproterenol (0 1 μg/min) Employing appropriate controls he found that a four-fold increase in mucosal perfusion to the chambered gastric segment would provide complete protection against aspirin injury (Table I) This was accompanied by a somewhat lesser rate of H^+ back-diffusion as compared to the mucosae that developed erosions with normal levels of mucosal blood flow These interest ing results are very new and as yet have not been fully appreciated as to their full significance It would appear however that enhanced mucosal blood flow in some way interferes with the usual barrier disruptive potential of aspirin

TABLE I BLOOD FLOW AND HYDROGEN ION LOSS FOR EACH 15-MIN PERIOD AFTER ASPIRIN INJURY

LESION	H^+ Loss μeq/min	Blood Flow (ml/min)
NONE (14 periods in 3 dogs)	17 3 ± 2 2	36 3 ± 3 4
MILD (19 periods in 4 dogs)	21 7 ± 1 3	26 5 ± 3 1
SEVERE (24 periods in 6 dogs)	22 3 ± 1 7	8 8 ± 0 8

DISCUSSION

The following observations are quite firm 1) *Barrier breakers such as* aspirin alcohol and bile alts produce an increased rate of H^+ back diffusion and Na^+ efflux with mild to severe mucosal lesions There is also a significant increase in mucosal blood flow but of a degree less than that of the increase in ion exchange 2) Barrie breakers that produce a large flux of H^+ and Na^+ and a comparable change in blood flow are not accompanied by significant mucosal injury 3) A decrease in mucosal blood flow in the presence of increased back diffusion of H^+ following exposure to a mild barrier breaker

leads to severe mucosal injury 4) Supernormal levels of mucosal perfusion prevent aspirin injury and the usual marked increase in H^+ back-diffusion associated with this agent These results demonstrate an intimate relationship between mucosal blood flow and acid injury Clearly a decrease in blood flow in the presence of a rapid rate of H^+ permeation leads to erosive gastritis Substances which are accompanied by an increase in perfusion that is comparable to the increment in H^+ back-diffusion are protected against significant injury It is likely in this instance that the increased blood flow helps to dispose of H^+ ions either by buffer dilution or other processes that allow the surface cells to withstand exposure to increased penetration by H^+ ions

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Acid base Balance in the Frog Gastric Mucosa, Role of the Extracellular Space

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ABSTRACT: Equilibrium of acid and base was measured in the extracellular compartments of *in vitro* frog gastric mucosa; 5 5-dimethyloxazolidine-(2 4-dione-2-C^{14}) was used as weak acid and nicotine-H^3-(G)-bitartrate was used as weak base Inulin-carboxyl (carboxyl-C^{14}) or Inulin(H^3) were used in the same mucosae to measure the serosal and mucosal extracellular volumes It is proposed: a) the existence of charges capable to maintain assymmetries in the distribution of electrolytes in the absence of acid secretion and b) the existence of restrictions between the extracellular spaces and the solutions able to develop differences in the electrolyte concentrations as consequence of the pH differences during acid secretion

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The distribution of weak electrolytes has been used to estimate the intracellular pH in different tissues (1) The fundamental assumption of the intracellular pH measurement is that the undissociated form of the indicator compound passes freely across cellular membranes by passive diffusion and reaches equal concentrations on the two sides The concentration of the ionic form will be determined by the concentration of the undissociated form and by the pH Using this principle the intracellular pH can be calculated if the compound can be analitically determined in the intracellular and extracellular water This is valid when the extracellular concentration and pH are known and the intracellular concentration measured

It is generally considered that the solution is in direct

contact with the cell membrane The continuous cell layer covering the frog stomach is mainly formed by epithelial and oxyntic cells (2) In the preparation called *in vitro* frog gastric mucosa only the surface epithelial cells which represent 1/7 of the cell area are directly exposed to the secretory solution (3) The other epithelial cells and the oxyntic cells are in the glands The glands pits and tubules form the extracellular space In this space a hydrogen ion accumulation is expected due to the primary acid secretion At the serosal surface the intercellular spaces between the lateral cell membranes and the cell surfaces in contact with the basement membrane are separated from the nutrient solution by the lamina propia the muscularis mucosa and part of the submucosa It has been proposed that in this serosal extracellular space a substantial reduction in the carbon dioxide simultaneously with a bicarbonate increase takes place during acid secretion (4 5) However no direct measurements of the extracellular spaces composition have been made and consequently neither the composition of extracellular content nor the nature of the restriction between these spaces and the solutions are known

It seems possible to use the kinetics of the distribution of weak electrolytes to measure their activities in the extracellular compartments

The present contribution is an attempt to measure the asymmetry in the distribution of weak electrolytes in the extracellular spaces of the acid secreting and inhibited mucosae No attempt to estimate pH values for the extracellular or cellular volumes is made Washout kinetics of 5 5-dimethyloxazolidine-(2 4-dione-2-C^{14}) and Nicotine-H^3-(G)-d-bitartrate across the two surfaces were done

Rana pipiens were used in these experiments The 7 4 pH solution previously described (3) was buffered with 17 8 mM $NaHCO_3$ and 5% CO_2 The $NaHCO_3$ concentration was changed to 5 8 and 57 5 mM to prepare the 6 9 and 7 9 pH solutions These solutions were also equilibrated with O_2-CO_2 95-5 In both cases the NaCl concentration was changed to keep the

total sodium constant and the osmolality between 218 and 225 mOsm/Kg water Acid secretion was stimulated with 10^{-4} M histamine diphosphate or inhibited with 2×10^{-3} M sodium amytal.

The method used in the experiments is summarized in Table I

TABLE I

Experimental Procedure

(1) Gastric mucosa preparation obtained by blunt dissection

(2) Loaded in double labelled solution (90 min)

(3) Mounted between two chambers and washed (120 min)

(4) Mucosa weighted and extracted (48 h)

(5) Samples from steps 2 3 and 4 counted for H^3 and C^{14} activities

The mucosae were loaded for 90 min in buffered solution labelled simultaneously with DMO-C^{14} and Inulin-H^3 or with Nicotine-H^3 and Inulin-C^{14} After blotting each mucosa was mounted as a flat sheet between two symmetrical chambers Both chambers were filled with buffered unlabelled solution of the same pH used to load the mucosa The unlabelled solutions in both chambers were totally replaced at 5 10 15 20 25 30 40 50 60 and 90 min Samples of the double labelled loading solutions the unlabelled washout solutions and the extract obtained from mucosa after 48 hours incubation and 40 C were counted for H^3 and C^{14} activities Extract of unlabelled mucosae was added to prevent quenching differences due to proteins (6) The activity remaining in the mucosa at each time was obtained by adding the activities recovered in the solutions placed in the chambers after the corresponding time

Table II presents the equations used to fit the activity remaining in the tissue as function of the washout time The two-exponential model was chosen because in a three-exponential model previously used the third exponential represents less than 0 005 of the total activity of the mucosa (7)

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that the tracer is in the same concentration of the solutions or even that the tracer is homogeneously distributed in the extracellular compartments

TABLE III

DMO Distribution in the Tissue Water

	Activity in the tissue water / Activity in the loading solution	
pH	Serosal surface	Mucosal surface
6 9	1 34 ± 0 06	0 33 ± 0 03
	0 84 ± 0 05	0 29 ± 0 03
7 4	1 05 ± 0 05	0 23 ± 0 02
	0 78 ± 0 04	0 25 ± 0 03
7 9	0 87 ± 0 03	0 20 ± 0 03
	0 61 ± 0 04	0 15 ± 0 02

Upper values were obtained in histamine stimulated mucosae and lower values were obtained in amytal inhibited mucosae

The upper values correspond to the results obtained in the histamine stimulated mucosae The lower values correspond to the amytal inhibited mucosae DMO activity in the serosal extracellular compartment is reduced by amytal inhibition of the acid secretion The differences are significant (P 0.001) at the three pH values studied No significant difference is observed at the mucosal surface by effect of amytal inhibition This suggests that at least part of the DMO accumulation at the serosal extracellular compartment is related to the CO_2/HCO_3^- change produced during acid secretion In addition there are at both surfaces significant DMO accumulation observed in the amytal inhibited mucosae

Table IV presents the nicotine recovered from the extracellular compartments Nicotine accumulates in the mucosal extracellular compartment in histamine stimulated mucosae No significant accumulation is observed in the serosal extracellular compartment The amytal inhibition of the secretion simultaneously reduces the nicotine accumulation in the mucosal extracellular space and increases its activity in the serosal extracellular space But even in the amytal inhibit-

ed mucosae there exist significant differences between the mucosal and serosal extracellular nicotine activites

TABLE IV

Nicotine Distribution in the Tissue Water

	Activity in the tissue water / Activity in the loading solution	
pH	Serosal surface	Mucosal surface
6 9	0 38 ± 0 04	0 58 ± 0 05
	0 81 ± 0 11	0 55 ± 0 06
7 4	0 56 ± 0 11	1 53 ± 0 33
	1 02 ± 0 14	1 19 ± 0 14
7 9	0 56 ± 0 08	2 25 ± 0 27
	0 71 ± 0 10	1 19 ± 0 19

Upper values were obtained in histamine stimulated mucosae and lower values were obtained in amytal inhibited mucosae

Simultaneously with the electrolyte activity changed produced during inhibition changes in total tissue water and inulin spaces have been reported (6)

TABLE V

DMO Distribution in the Extracellular Compartments

	Activity in the extracellular water / Activity in the loading solution	
pH	Serosal surface	Mucosal surface
6 9	4 50 ± 0 60	3 01 ± 0 82
	2 06 ± 0 11	5 18 ± 0 39
7 4	2 12 ± 0 11	3 96 ± 0 31
	2 10 ± 0 28	3 84 ± 0 49
7 9	2 05 ± 0 13	2 08 ± 0 21
	1 97 ± 0 27	2 63 ± 0 25

Upper values were obtained in histamine stimulated mucosae and lower values were obtained in amytal inhibited mucosae

Table V presents the same DMO extracellular activities previously shown but this time referred to the extracellular water volume The extracellular water volume was es-

timated from the tissue water and its fraction reached by inulin from the serosal and the mucosal surfaces

DMO significantly accumulates at both the serosal and the mucosal surfaces Only at 6 9 pH the activity reduction at the serosal surface due to amytal inhibition remains significant No differences could be observed in the other five couples of concentration values This suggests that extracellular DMO and water change simultaneously with amytal inhibition

TABLE VI

Nicotine Distribution in the Extracellular Compartments

	Activity in the extracellular water / Activity in the loading solution	
pH	Serosal surface	Mucosal surface
6 9	0 92 ± 0 12	10 3 ± 3 4
	1 56 ± 0 17	10 6 ± 1 2
7 4	1 14 ± 0 24	34 2 ± 7 4
	2 63 ± 0 40	28 8 ± 5 4
7 9	1 18 ± 0 12	29 3 ± 2 8
	1 71 ± 0 27	21 3 ± 5 4

Upper values were obtained in histamine stimulated mucosae and lower values were obtained in amytal inhibited mucosae

Table VI presents the nicotine extracellular activities referred to the extracellular water volumes The nicotine accumulation in the serosal extracellular space becomes significant during amytal inhibition suggesting that water increase is smaller than nicotine accumulation (P 0 001) Nicotine accumulation at the mucosal extracellular space is re duced significantly (P 0 001) Using the values reported in the last two figures which are referred to the extracellular water volume it is possible to estimate the activity ratios between the mucosal and serosal extracellular contents

In the absence of acid secretion when the net chloride flux is also reduced it is clear from Table VII that both the DMO that ionizes as a weak acid and the nicotine that ionizes as a weak base accumulate significantly more in the

mucosal negative extracellular space than in the serosal positive extracellular space In this condition no pH difference exists between the surfaces able to explain the assymmetrical distribution of the electrolytes

TABLE VII
Serosal to Mucosal Ratios

	5 5-Dimethyloxazolidine 2 4-dione-2-^{14}C	
pH	*Stimulated mucosae*	*Inhibited mucosae*
6 9	0 78 ± 0 17 (14)	2 60 ± 0 24 (10)
7 4	1 87 ± 0 15 (22)	2 12 ± 0 38 (10)
7 9	1 11 ± 0 15 (16)	1 53 ± 0 22 (10)
	Nicotine-H -(G)-D-bitartrate	
pH	Stimulated mucosae	Inhibited mucosae
6 9	13 5 ± 4 7 (12)	8 7 ± 2 5 (10)
7 4	33 2 ± 4 8 (10)	11 0 ± 1 0 (10)
7 9	28 2 ± 5 2 (10)	12 6 ± 2 4 (10)

Each value is the mean ± SE In parenthesis are the number of experiments

Histamine stimulation of acid secretion towards the mucosal surface and bicarbonate release towards the serosal surface induces the expected increment in the mucosal to serosal nicotine activity ratio and the corresponding reduction *in the mucosal to serosal DMO ratio*

In short the measured distribution of weak acid and base in the extracellular compartments of the *in vitro* frog gastric mucosa during inhibition and stimulation of acid secretion suggest: (a) the ability to maintain assymmetries in the distribution of electrolytes in the absence of acid secretion; (b) the existence of restrictions between the extracellular spaces and the solutions able to develop differences in the electrolyte concentrations as consequence of the pH differences during acid secretion; and (c) that in these conditions the measurements of total weak electrolytes distribution does not represent measurements of the pH of the

extracellular or the pooled cell compartments

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The Effects of K^+ and Na^+ on Acid Formation in Isolated Gastric Glands

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ABSTRACT Isolated gastric glands from the rabbit incubated in K^+ free medium lost their ability to respond to secretagogues both in terms of oxygen consumption as well as aminopyrine(AP) accumulation Also the basal AP accumulation was totally abolished The K^+ content of the glands could not be lowered more than 50% Addition of K^+ to glands pre treated with db-cAMP induced rapid metabolic and secretory responses Increasing the K^+ concentration to 54mM did not change the basal oxygen consumption but gave a higher AP accumulation Incubation in Na^+ free medium with standard K^+ concentration made the glands lose most Na^+ and 2/3 of their K^+ content In spite of this the glands responded in a normal way to secretagogues It is suggested that K^+ but not Na^+ is essential for the H^+ secretion that K^+ highly couples the secretion and the metabolism and finally that extracellular K^+ is the primary source for the K^+ involved in acid secretion

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INTRODUCTION The ionic requirements for acid secretion has been extensively studied in the isolated amphibian gastric mucosa and K^+ Cl and Ca^{++} has been shown to be essential for the acid secretory mechanism (1 2 3) Contradictory reports exist about the importance of Na^+ (4 5)

In this respect little has been known about the conditions necessary for the mammalian parietal cell due to the lack of a sensitive isolated system Now however several working mammalian preparations have been presented one of which is the preparation of isolated gastric glands (6) The glands obtained from the rabbit gastric mucosa have now been characterized with respect to their basic properties their responsiveness to

secretagogues and inhibitors their morphology in resting and stimulated state and more recently the importance of Cl for the formation of acid in the glands was demonstrated (7 8 9)

The topic of the present investigation was to explore the requirement of K^+ and Na^+ for the formation of acid in these isolated gastric glands

MATERIAL AND METHODS: Isolated glands were obtained by the method of Berglindh and Obrink (6) After separation the glands were divided into different fractions and each washed in their respective medium 5 times during 45 min at room tempe ture

Solutions Normal incubation medium NaCl 132 4mM KCl 5 4mM NaH_2PO_4 1 0mM Na_2HPO_4 5 0mM $MgSO_4$ 1 2mM Ca acetate 1 0mM pH 7 4

Na free medium Choline chloride 140 0mM KH_2PO_4 6 0mM $MgSO_4$ 1 2mM Ca-acetate 1 0mM pH was adjusted to 7 4 with a few drops of Tris base (300mM)

K^+ free medium was obtained by replacing KCl by NaCl

High K^+ medium (54mM) was obtained by replacing NaCl by an equivalent amount of KCl All media contained 2mg/ml glucose 2mg/ml albumin (Sigma) and 0 001% phenol red

Chemicals Db-cAMP as sodium salt histamine diphosphate and aminophylline were obtained from Sigma Aminopyrine (N-4-dimethyl-C14) with a specific activity of 19mCi/mmol was a product of NEN Chemicals Inulin (C14) carboxylic acid with a specific activity of 0 8mCi/mmol was obtained from the Radiochemical Center Amersham

Glandular parietal cell response was studied by means of changes in oxygen consumption and accumulation of aminopyrine (AP)(the latter is a weak base which will be trapped in acid compartments) Both methods have been described thoroughly elsewhere (7,8)

The electrolyte content of the glandular cells was determined by

using Inulin-C14 as extracellular marker Dried pellets of glands were dissolved in 0 2ml HNO_3 and subsequently diluted to an appropriate con centration by addition of water Na^+ and K^+ were determined in an Eppendorf flame photometer

Statistical analysis was performed using the Student s t test for paired samples

RESULTS Glands of the same population were incubated in normal K^+ free as well as in a medium containing 1mM K^+ the latter was obtained by mixing proper amounts of normal and K^+ free medium To each Warburg flask 0 5μCi AP was added Each group of glands was either untreated or stimulated by histamine 10^{-4}M or db-cAMP 10^{-3}M

After 30 min preincubation at 37 C oxygen consumption was studied for 60 min at which time the AP accumulation ratio (i e AP in intraglandular water/AP in extraglandular water) was determined The respiratory rates as well as the AP accumulation ratios obtained in this way are listed in Table I

The glands incubated in the normal medium showed the standard response to addition of histamine or db-cAMP in terms of increased oxygen consump- tion and AP accumulation (7) In the K^+ free medium the basal oxygen consumption was not significantly altered but the glands lost their ability to increase the respiratory rate upon addition of the secreta gogues Normally unstimulated glands showed a considerable basal AP accumulation (7 8) Without K^+ in the medium this accumulation was al most totally abolished thus it decreased from 43 6 to 2 6 This low basal value was not significantly affected by histamine but did increase to 17 0 in the presence of db-cAMP

With 1 0mM K^+ in the medium the glands showed a small metabolic and secretory responsiveness to secretagogues Also the basal accumulation

secretagogues and inhibitors their morphology in resting and stimulated state and more recently the importance of Cl for the formation of acid in the glands was demonstrated (7 8 9)

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The electrolyte content of the glandular cells was determined by

and oxygen consumption since no time consuming morphological change has to occur

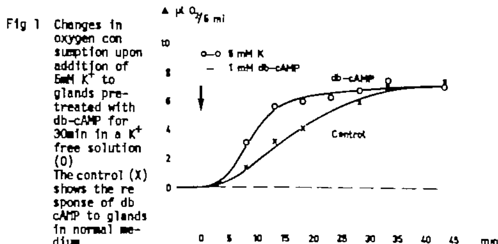


Fig 1 Changes in oxygen con sumption upon addition of 5mM K^+ to glands pre-treated with db-cAMP for 30min in a K^+ free solution (O) The control (X) shows the re sponse of db cAMP to glands in normal me-dium

Fig 1 shows such an experiment where the oxygen consumption was mea sured in glands pretreated with db-cAMP in a K^+ free solution The ad dition of 5 0mM K^+ resulted in a rapid increase in oxygen consumption up to a new steady state within 15 min Simultaneously glands incubated in normal medium was given 1 0mM db-cAMP and they followed the same stimula tory time-response pattern as seen before (7) i e 35-40 min was needed to obtain a steady state value

The same type of experiment was performed with AP accumulation Glands incubated in a K^+ free medium were divided in three portions To one db-cAMP 1 0mM was added at time 0 and all three were incubated for 45 min At this point 5 4mM K^+ was added to the db-cAMP treated and to one of the untreated while the third received both 5 4mM K^+ and 1 0mM db-cAMP The incubation then continued for another 45 min A typical example of such an experiment is shown in Fig 2 The glandular AP accumulation was fol lowed by determination of the extraglandular AP content which is a sen-

Table III

Electrolyte and water content in glands incubated in different media during 45 min The glands were not obtained from the same preparation except for the Na^+ free Mean ± S E is presented

Medium	$[Na^+]$ mEq/kg ICW	$[K^+]$ mEq/kg ICW	kg ICW/kg dry wt
Normal n 9	23 2 ± 3 8	133 7 ± 2 3	2 04 ± 0 06 from ref (6)
K^+ free n 6	95 8 ± 5 3[a]	64 3 ± 2 1[a]	2 00 ± 0 08
54mM K^+ n = 5	11 2 ± 1 7	147 5 ± 6 4	1 89 ± 0 13
Na^+ free n 5	4 4 ± 0 1[a]	38 0 ± 0 5[a]	2 38 ± 0 02[a]
Na^+ free + 1mM db-cAMP n 5	3 9 ± 0 1[a b]	32 2 ± 0 4[a b]	2 67 ± 0 01[a b]

a -denotes values significantly different (P<0 05) from the normal medium
b—denotes values significantly different (P<0 05) between the Na^+ free

Na^+ free medium Glands incubated in choline medium with normal K^+ concentration had a basal oxygen consumption which was 25% below the control value Also the basal AP accumulation ratio was significantly decreased In Table IV is also listed the glandular responses to histamine 10^{-4}M aminophylline 10^{-3}M and db-cAMP 10^{-3}M In contrast to the K^+ free situation the incubation in Na^+ free medium did not inhibit the stimulatory effect of the secretagogues On a percentage basis the increase above

Table IV

Oxygen consumption and aminopyrine (AP) accumulation ratio in normal and choline incubated glands Each experiment was performed on glands from the same population n 4 except where noted Mean ± S E

	Normal medium		Na^+ free medium	
	µl O_2/mg dry wt x 30min	AP rati ICW/ECW	µl O_2/mg dry wt x 30min	AP rati ICW/ECW
Control	7 9±0 6	27 7±4 3	5 9±0 4[b]	13 2±3 8[b]
Histamine 10^{-4}M	10 8±0 8[a]	57 5±8 1[a]	8 4±0 8[a b]	62 9±11 6
Aminophylline 10^{-3}M	8 1±0 7[a] n=3	74 1±8 2[a] n=3	9 7±1 2[a] n=3	102 0±17 4[a] n=3
Db-cAMP 10^{-3}M	13 1±1 9[a]	85 4±10 8[a]	12 0±1 4[a]	140 5±19 1

a—denotes values significantly different ($P<0\ 05$) from the respective control
b—denotes values where the same treatment gave a response significantly different ($P<0\ 05$) from that of the normal medium

basal in both oxygen consumption as well as in AP accumulation was in fact higher than for the normal glands In absolute figures only the histamine stimulated respiration in Na^+ free glands was significantly lower than the corresponding control Aminophylline in particular gave differing results in terms of oxygen consumption thus in normal medium the increase was only 2 6% whereas a 64 6% increase was seen in the Na^+ free situation Very high figures were also obtained for the stimulated AP accumulation and they did not significantly differ from those found in the normal glands

ly lowered

Db-cAMP in K^+ free medium The addition of db-cAMP to glands in K^+ free medium caused a small but significant increase in the AP accumulation This could either be interpreted as a small stimulation of acid formation or since no metabolic response was seen as an increase in AP accumulation space (8) The latter would indicate a morphological transformation of the parietal cells from a resting to a stimulated state (7 8) The rapid response obtained upon addition of K^+ to glands pretreated with db-cAMP (Fig 1 2) indicated that the glands by some means had been prepared for secretion whether by a morphological change and/or by some other means must be further explored

In any case the intracellular pool of transport K^+ must have been reestablished possibly indicating a direct pathway for the K^+ to that pool In addition K^+ might directly activate the K^+-ATPase in the secretory membrane of the parietal cells via the lumen of the gland provided the cells are in a canalicular state This would correspond to the instant return of secretion seen when K^+ is added to the secretory side of the frog gastric mucosa (1)

Metabolic secretion coupling It is noteworthy that the glands in the absence of extracellular K^+ lose both the ability to form acid as well as the ability to increase the metabolic rate upon addition of secretagogues This is in contrast to the behavior of glands incubated in Cl free medium where the glands responded with an increase in oxygen consumption although the AP accumulation was very poor (9) Thus K^+ seems to be crucial for the direct coupling between acid formation and metabolism a point which is further supported by the parallel increase in oxygen consumption and AP accumulation in the case discussed above Accordingly in the absence of K^+ the process o cascade of processes leading to H^+ secretion is

prevented This sharply indicates that the K^+-activated ATPase might be one of the terminal steps in the H^+ secreting mechanism

High K^+ medium An increase of the extracellular K^+ concentration did not affect the basal oxygen consumption but increased the AP ratio Since there is no reason to believe that an increase in K^+ would induce a *morphological change of the parietal cells the increase in AP accumula* tion must be interpreted as an increase in acidity within the same space Histamine increased the respiratory rate but this was not coupled to an additional increase of the accumulation This is however not surprising since according to the theory of AP accumulation (8) the final accumula tion is the product of acidity and accumulation space

In conclusion 1 Extracellular K^+ is the main source for the K^+ involved in H^+ secretion in isolated gastric glands regardless of the K^+ content of the cells 2 Stimulation of the Na^+ free glandular parietal cell facilitated the access of transport K^+ to the secretory site 3 K^+ tightly couples secretion and metabolism 4 The K^+ involvement in acid secretion is one of the final steps

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Histamine, Cyclic AMP and the Secretory Response of Piglet Gastric Mucosa

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ABSTRACT The involvement of cycli 3 5 -monophosphat adenylat (c AMP) a an intracellular mediator during stimulation of H^+ secretion in mammalian g stric mucosa was investigated We me sured transepi thelial potential difference (p d),resistance H^+ secretion and tissue AMP content in an active in vitro preparation the piglet gastric mucosa during resting and Histamine-stimulated conditions and then during return to the resting stat following tissue washout. During the first 5 15 minutes after timulation tissue c AMP and H^+ secretion increased while resistance and p d decreased However while H^+ secretion re istance and p d showed sustained changes the changes in c AMP were only temporary Our data show that the increase in AMP is ynchronous with the decre se in resistance and that these events precede H^+ secretion; once initiated the H^+ secretion as well as the decreased resistance are maintained even fter the c-AMP content has declined Tissue washout caused c-AMP p d resistance and H^+ ecre tion to return to resting levels within 45 minutes

INTRODUCTION

The secretagogue induced transition of the resting g stric mucosa to the actively secreting condition involves dramati changes in ultrastructure and lectrophysiological variables of the oxyntic (HCl-secreting) cell (1) For the amphibian g stric mucosa the con cept of adenosine 3 5 -monophosphat (-AMP) a second intra cellular mediator of these changes has been widely accepted For example methyl xanthines which inhibit the c AMP degrading enzyme phosphodiesterase stimulat H^+ secretion (2 3) and this increase in H secretion is well correlated with both the time course and lso magnitude f increased tissue content f AMP Although there seems to be little question that -AMP i intimately involved in H^+ secre

tion (though the actual step or steps involved in this complex process which are c-AMP sensitive are still unknown) by frog gastric mucosa there has been little agreement regarding its role in H^+ secretion by mammalian stomachs Exogenously added c AMP and theophylline has been reported to cause increased H^+ secretion in vitro rabbit stomach (4) but a decrease in vivo rat stomach (5) and no effect in vivo dog stomach (6) Biochemical assays have shown the presence of an adenyl cyclase in many species of mammalian gastric mucosa but the sensitivity of this enzyme to secretagogues has been disputed Perrier and Lester (7) observe a stimulation by histamine but Jacobson s group (8) does not It is not surprising that two recent reviewers of this topic have come to opposite conclusions Kimberg (9) suggested that H^+ secretion is correlated with increased levels of c AMP while Amer (10) cautiously suggested that the correlation is with decreased levels of c AMP

This controversy led us to design experiments in which we measured c AMP content and electrical and secretory variables all at the same time in one in vitro piglet gastric mucosa both during histamine stimulation and then during return to resting state following wash-out of tissue In vitro piglet gastric mucosa has proven to be an exceptionally useful mammalian preparation for several reasons: (1) It can be easily and rapidly removed from underlying muscle and connective tissue layers; (2) it responds rapidly to histamine addition and removal; (3) in contrast to other in vitro preparations of mammalian stomach the secretory and electrical responses are very similar to those observed in vivo and these can be maintained for up to 6 hours (11); (4) the complications inherent to in vivo preparations e g changes in blood flow are circumvented

MATERIALS AND METHODS

Gastric mucosae from baby pigs (newborn to 19 days of age) were prepared for in vitro experiments in a manner which has been described in detail elsewhere (11) Briefly the animal was killed by a blow to the head and subsequent transection of the spinal cord The stomach was quickly removed and the gastric mucosa separated from the serosal muscle coats by a "blistering technique Two separate pieces of the epithelium were then tied onto the end of mounting tubes (1 4 cm^2 area) which were i turn quickly assembled into separate

water jacketed (37°C) Ussing type chamber (see Fig 1 of ref 11) Warmed bathing solutions were introduced and gassing with 95% O_2 5% CO_2 commenced immediat ly The serosal bathing solution contained (in mM) 122 NaCl 25 $NaHCO_3$ 5 KCl 1 $MgCl_2$ 1 $CaCl_2$ and 10 glucose while the mucosal bathing solution wa 150 mM NaCl

Transepithelial lectrical potential difference (p d) was measured by connecting the bathing solutions through 1 M NaCl-agar bridge and calomel half cells to a high impedance lectrometer and strip chart recorder The sign of the p d wa always negative mucosal side with respect to serosal side Resistance was measured using voltage clamping device by measuring the current required to clamp the p d to a value 5 mV less than the spontaneous open circuit p d The piglet gastric mucosa has a linear ohm-c resistance over this range of p d. s (12) Since the resistance of the solutions (12 to 28 Ω cm^2) was not insignificant compared to that of the tissue all reported resistances have been corrected for this solution re istance Rates of H^+ secretion were monitored by the pH stat technique of Durbin and Heinz (13): the mucosal fluid was maintained at pH 5 0 by addition of 0 1 N NaOH (Radiometer Autoburette) which was measured at 3 minute intervals

Tissue c AMP content was measured by a method modified from Gilman (14) The pieces of ti sue were placed in a small glass homogenizer containing 1 0 ml of ice-cold 50% glacial acetic acid Aft r homogenization 100 ul sample was further precipitated in 10% ice cold trichloroacetic acid for determination of prot in content by the method f Lowry (15) The rest of the homogenate (900 ul) was spun for 4 minutes in an Eppendorf centrifuge and 800 ul of the supernatant was dried in a vacuum oven This dried extract was then dissolved in 200-250 ul of 50 mM Tris-Cl (pH 7 5) containing 4 mM EDTA A 50 ul aliquot was assayed in duplicate for c-AMP using -AMP Analysis Kit from Amersham/Searle

The specific experimental protocol wa as follows: Two piece of the same gastric mucosa were mounted in the chambers nd allowed to equilibrat over the cours of approximat ly 45 minutes During this time the p d and resistance both increased and stabilized at approximately -45 mV and 125 Ω cm^2 respectively H^+ secretion was basal or occurred at very low rat s (≤ 0 4 eq /cm^2 hr) in the non

stimulated tissues In these experiments there as some variability from preparation to preparation Preliminary experiments indicated that resting tissues with resistances less than 100 $\Omega\text{-cm}^2$ were unresponsive to histamine stimulation We have therefore used only those tissues which had resting resistances greater than 100 $\Omega\text{-cm}^2$ At specified times after histamine stimulation or its removal one of the chambers was rapidly disassembled and portion of the tissue was cut out for analysis of c AMP content This procedure took approximately 7 seconds Electrical variables and H^+ secretion were monitored in the companion piece of tissue Thus p d resistance H^+ secretion and c AMP content could ll be analyzed in the same tissue during resting conditions at difi rent times after histamine addition and then during the return to resting conditions after the removal of histamine addition and then during the return to resting conditions after the removal of histamine from the solutions (wash 4 times with fresh solutions) We also performed control experiments to assure ourselves that the changes in c AMP content were not artifacts due to contamination from any residual connective tissue and/or smooth muscle In two experiments the serosal muscl layer which had been dissected off the gastric mucosa was incubated under identical conditions as those described for the gastric mucosa and the c-AMP content was measured after histamine addition over the same time course of incub tion as that of the gastric mucosa

As we were interested in the time sequence of changes in c AMP content as well as in electrical and secretory variables and because of the vari bility from tissue to tissue for the analysis of the data we have chosen to normalize them in the following manner: During stimul tion we calculated for each tissue and t each time (0 5 10 30 and 45 minutes) fter histamine stimulation the ratio between changes at given time and maximal change occurring during the 45 minutes stimulation Thi w s done for each variable As an example the ratio for changes in the c AMP content is given by the formul :

$$\frac{\Delta c\text{-AMP}_t}{\Delta\ \text{AMP}_{max}} \qquad \frac{c\text{-AMP}_t \quad c\text{-AMP}_0}{c\text{-AMP}_{max} \quad \text{-AMP}_0}$$

where $c\text{-AMP}_0$ r rs to the cont nt during resting conditions $c\text{-AMP}_t$ to that measured t t minutes after stimul tion nd -AMP_{max} to the maximum value d n, the 45 minute stimulation; $c\text{-AMP}_t$ and $c\text{-AMP}_{max}$

therefore refer respectively to the change in c-AMP content at time t and the maximum change which was me sured during the experiment Similar ratios were c lculated for the tissue wash-out experiments except that here t=0 refers to the histamine stimulated stat Thus the re istance ratio w s calculated using the formula bove while for c AMP and H^+ because the value at t=0 lso is the maximum value the ratios reduce to $\frac{c\ AMP_t}{c\ AMP_0}$ and $\frac{H_t}{H_0}$ respectively

To visualize how the different variables were changing in time compared to e ch other we constructed phase diagrams in which we plotted the normalized value for any other vari ble at a given time t If two variables are synchronous the experiment points should lie along the line of identity (45^0 slope); if two vari bles are not related in time the points will lie either above or below the line of identity

RESULTS

Histamine stimulation

Addition of 5×10^{-5} M histamine to the serosal solution of piglet gastric mucosa caused rapid chang in transepitheli l resistance and AMP levels and somewhat delayed H^+ s cretion Immediate fluctuations in p d lso usually occurred ($^+$ 2 3 mV) within 5 seconds The averag responses (-SEM) of 11 these variables are shown in Table I The chang in electrical and secretory vari bles are quite dramatic and indicat that thi *in vitro* preparation i physiologically active The rates of H secretion (up to 15 ueq/cm^2.hr in the s series of experiment) are the highest recorded for any mammalian preparation *in vitro*

TABLE 1

RESTING AND HISTAMINE-STIMULATED PIGLET GASTRIC MUCOSA

	c AMP pmole/mg protein	Resistance Ω cm^2	H secretion ueq /cm^2 hr	p d mV	N
Resting	1 7 + 0 5	120 + 8	0 4 + 0 4	44 1 + 2 3	7
Minutes after histamine stimulation					
5	26 6 10 2	80 + 6	2 1 + 0 1	43 0 + 1 8	7
10	30 4 + 15 3	74 + 5	6 6 + 0 2	41 6 + 1 8	7
15	18 9 + 6 7	76 + 6	7 9 + 0 2	40 7 + 1 7	7
30	19 1 ± 13 6	75 + 5	8 9 + 0 2	36 1 + 2 6	3
45	5 2 + 1 1	68 + 2	8 9 + 0 02	42 0 + 0 5	7

Values are expressed as mean + SEM

Several features are of interest in Table 1: (1) c AMP content was low until addition of histamine; (2) after 10 minutes of histamine stimulation the c AMP content increased by an average of more than 15 fold; (3) resistance decreased and H^+ secretion increased whil p d changed only slightly over the same time period; (4) at times greater than 15 minutes the c AMP content declined while H^+ secretion resistance and p d remained fairly constant Since the c AMP content in serosal muscle tissue remained constant (2 0 3 0 pmole/mg protein) during the whole incubation time we attribute changes in the c AMP content as well as those in secretory and electrical variables to activities of the gastric mucosa

We have further analyzed the experimental data by normalizing them according to the procedure described in Materials and Methods; the results are depicted in Figure 1 (a f) The maximum change in c-AMP content occurred within 5 minutes (Fig 1a) while the maximum increase in H^+ secretion was delayed until 30 minutes (Fig 1c) The phase diagram (Fig 1d) shows that during stimulation changes in c-AMP content preceded changes in H^+ secretion and that after 30 45 minutes there was no correlation between c AMP content and H^+ secretion Figure 1e shows that during the first 10 minutes the changes in c-AMP content slightly precede changes in resistance but that the two are nearly synchronou As in the case of H secretion after 30- 45

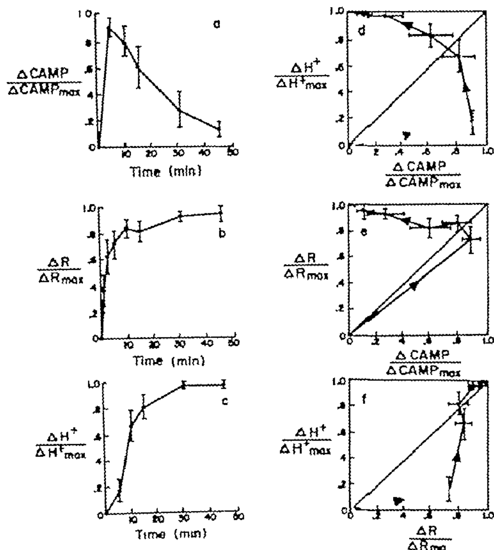


Figure 1 Histamine stimulation
are the mean normalized values $^{+}$ standard error of the mean for change in AMP content resistance and H secretion respectively as they proced in time after stimulation with histamine 5 x 10^{-5} M at t = 0 d f are the phase diagrams; (d) c-AMP content versus H^{+} secretion; (e) AMP cont nt versus resistance; and (f) resist nce versus H^{+} secretion The arrows how the progression of time (t) with t=0 at 0/0 nd t=45 at 0/1 0 for 1d and 1e and at 1 0/1 0 for 1f The line f identity is the line with 45^{0} slope

minutes changes in resistance and c AMP content are not related to each other Figure 1f shows that while changes in resistance precede H^+ secretion up to 15 minutes after stimulation the two events are synchronous from 15 minutes on

Tissue wash-out

In five further experiments we stimulated paired halves of gastric mucosae with histamine (5×10^{5} M) and allowed them to incubate for 45 minutes while monitoring the above variables Then after cutting out a control piece in one of the halves for the measurement of c AMP content both halves were washed four times with histamine free solutions

T BLE 2

TISSUE WASH-OUT

	c AMP pmole/mg protein	Resistance Ω cm^2	H^+ secretion ueq /cm^2 hr	p d mV	N
Histamine stimulated for 45 min	7 0 $\pm$ 2 9	70 5 $\pm$ 4 8	9 4 $\pm$ 1.2	28 6 $\pm$ 4 2	5
Minutes after histamine removal					
10	6 2 $\pm$ 1.8	90 4 $\pm$ 10 8	6 9 $\pm$ 1 8	22 4 $\pm$ 4 3	5
20	2 8 $\pm$ 0 5	106 5 $\pm$ 7 4	2 0 - 1.2	16 9 $\pm$ 3 4	5
45	2 1 $\pm$ 0 5	115 0 $\pm$ 17 7	0 4 $\pm$ 0 4	19 5 $\pm$ 4 3	5

Values are expressed as mean SEM

Table 2 shows that during the wash-out resistance H^+ secretion and c AMP content all slowly returned to their resting levels The p d which had dropped slightly during stimulation declined even further We attribut th drop in c AMP content to two factors: (1) a lack of the stimulatory influence of histamine and (2) a loss of cellular c AMP due to diffusion through cell membranes (and breakdown of phosphodiest rase?)

Figure 2 show the changes in c AMP content (Fig 2a) H^+ secretion (Fig [illegible] re ist nce (Fig 2c) the changes in resistance and H secreti [illegible] fast and more pronounced than the changes

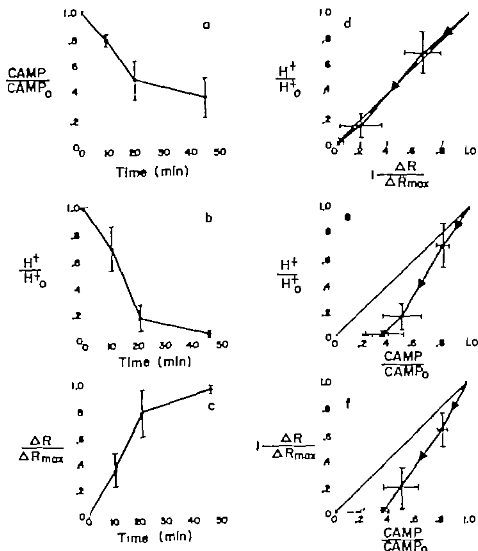


Figure 2 Tissue wash-out
are the mean normalized values $^{+}$ standard error of the mean for change in -AMP content H^+ secretion and resistance respectively as they procede in time after the removal of histamine (4 washes) at t=0 d f are the phase diagrams; (d) re istance versus H^+ secretion; (e) c AMP content versus H^+ ecretion; and (f) AMP content versus resistance The arrows show the progression of time (t) with t=0 at 1 0/1 0 and t=45 at 0/0 The lin of identity is the line with 45° slope

in c AMP content The phase diagrams show that during tissue wash-out decreases in H^+ secretion (Fig 2e) and increases in resistance (Fig 2f) both precede decreases in c AMP content Figure 2d on the other hand shows a very tight correlation between changes in resistance and H^+ secretion

DISCUSSION

An interesting aspect of this study was that with histamine in the bathing solutions the c AMP content first increased but then decreased such that after 30 45 minut s it had dropped to a level half (or less than) that observed at the peak (Tables 1 and 2) Despite this de crease in c AMP content H^+ secretion resistance and p d remained nearly constant In doing experiments of this type the time at which the c AMP content is measured is obviously an important variable A similar stimulant induced transient increase in c AMP con tent has been observed in several other tissues (e g neural tissue ref 16; pig epidermis ref 17)

These experiments demonstrate that over the course of the first 10 minutes after histamine addition changes in c-AMP content precede H^+ secretion (Fig 1d) but are roughly synchronous with decreases in resistance (Fig 1e)

The changes in H^+ secretion nd resistance observed during stimu lation are due to the activity of the oxyntic cells and it seems likely that the c AMP response is occurring in these cells too We cannot though completely discount the possibility that the chief (pepsinogen secreting) and/or surface-epithelial (mucus secreting) cells contribute to the c-AMP response

At times greater than 15 minutes because H^+ and resistance values remain constant at the same time as c AMP levels decline dramatically we conclude that steady elevated c AMP content is not the necessary intracellular factor determining H secretion and electrical changes in piglet gastric mucos A possible explanation for the results is that c AMP is somehow involved as a trigger for the movements and ultimate fusion of tubulo-vesicular acid secreting membranes from an intracellular location up to the apic l membrane (1) Once these membrane have been activated the presence of a high concentra tion of free AMP may not be required Thus the l rge peak of c-AMP content ft r stimul tion may be a necessary condition for

activation; the maintenance of the activated state may involve other agents either extracellular (g histamine) and/or intracellular (e g Ca^{2+} and/or protein kinase) at step distal to the initial for mation of c AMP

ACKNOWLEDGMENTS

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MATERIALS AND METHODS

Isolated fundic or antral mucosa from *Rana temporaria* or Necturus was used as described previously (3) Briefly the mucosa was separated t the rest of the stomach wall by blunt dissection in an unbuffered ... and mounted in Ussing chambers The area of the exposed muco- ... 9 or 1 3 cm^2 An unbuffered frog Ringer solution which was ... ith 100 % O_2 prewashed in $Ba(OH)_2$ to exclude possible traces ... s used on the luminal (secretory) side The pH on this side was ... tant at 7 40 by infusion of solutions containing 5 mM HCl or ... O I The amount of base or acid transported into the luminal ... calculated from the amount of HCl or NaOH infused The n... ...al) solution was buffered with HCO_3 (17 8 mM) and phos p... ...8 mM) and gassed with O_2 and CO_2 in a ratio of 95 5 (vol:vol) The standard nutrient and luminal solutions (3) always contained Na^+ 102 4 K^+ 4 0 Ca^{++} 1 8 Mg^{++} 0 8 and Cl^- 91 4 mM, SO_4^{--} and mannitol being added to isotonicity

To inhibit H^+ secretion fundic mucosae were always pre-treated with the histamine H receptor antagonist Metiamide (10^{-3}M nutrient side) until stable rates of net alkaline secretion were obtained and this drug was then present throughout the experiments Catecholamines were added only to the nutrient side and these solutions were prepared immediately before use Metiamide 17-GI-gastrin and pentagastrin were kind gifts from Dr M E Parsons SKF Herts England Dr M I Grossman Vet Adm Hosp Los Angeles Ca U S A and ICI-Sweden Gothenburg Sweden L-noradrenaline L-adrenaline and DL-isoprenaline were obtained from Sigma St Louis MO U S A

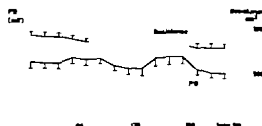
RESULTS

Stimulation by nutrient Ca^{++} The effects of elevation of the nutrient Ca^{++} (from 1 8) to 3 6 and 7 2 mM in the antral mucosa are shown in Fig 1 At the highest concentration Ca^{++} increased the alkalinization ($0.01 < p < 0.02$) and the electric potential difference ($p < 0.01$) but did not affect the transepithelial electric resistance The lower concentration of Ca^{++} had a similar but not significant effect ($p > 0.05$)

Abbreviation: PD transepithelial electric potential difference



Fig. 1. Effect of an increase in the nutrient Ca^{++} (from 1 8) to 3 6 or 7 2 mM on HCO_3 secretion and electrical properties of Necturus antral mucosa Means ± SE are given n = 6



An increase in alkalinization with 7 2 mM nutrient Ca^{++} also occurred in the fundic mucosa ($p<0$ 01 n = 8) In the fundus however the initial (30 min) part of the increase in alkalinization was masked by a transient increase in H^+ secretion The latter was not abolished by Metiamide

Table I Effects of pentagastrin and gastrin on HCO_3^- transport

	PD (mV)	Resistance (ohm cm^2)	OH (μeq cm^{2} h^{-1})	
Control	21 8 ± 2 1	407 ± 19	0 35 ± 0 05	
PG (10^{-6}M)	20 8 ± 2 1	409 ± 14	0 31 ± 0 04	(n = 11)
Control	19 9 ± 1 8	404 ± 19	0 28 ± 0 05	
Control	4 1 ± 0 4	853 ± 61	0 29 ± 0 05	
Gastrin (10^{-8}M)	3 4 ± 0 4	816 ± 61	0 26 ± 0 05	(n = 6)
Control	3 1 ± 0 3	789 ± 63	0 29 ± 0 05	

Fundic mucosa from Rana temporaria pre treated with Metiamide (10^{-3}M) was used in the experiments with pentagastrin (PG) and antral mucosa from Necturus in the experiments with gastrin The experiments were performed during three consecutive 45 min periods means ± SE during the last 15 min in each period are presented

Effects of pentagastrin and gastrin These agents had no effects on antral and alkalinizing fundic mucosae (Table I) Pentagastrin at the concentration used here (10^{-6}M) stimulated H^+ secretion in Rana temporaria fundus (n = 4)

Effects of catecholamines Noradrenaline inhibited fundic alkalinization in concentrations of 10^{-4} and 10^{-5}M (p<0 01 for both) but had no significant effect on the PD or electric resistance (Fig 2) A similar inhibition was observed in antrum (Fig 3) The effect was partially reversible on removal of the drug and could be prevented by pre treatment of the mucosa with phentolamine (Fig 4) Adrenaline inhibited fundic alkalinization but only at the highest concentration of 10^{-3}M (0 01<p< 0 02 n = 9) At 10^{-4} and 10^{-5} M adrenaline had no significant effect Isoprenaline did not affect the alkalinization (Fig 3)

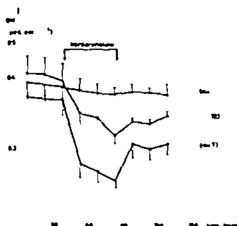


Fig. 2. Effect of noradrenaline on HCO_3^- secretion by Rana temporaria fundic mucosa The mucosae had been pre treated with Metiamide (10^{-3}M) until stable rates of alkalinization were obtained and this drug was then present throughout the experiments No changes in PD or resistance occurred in these experiments

The inhibitory effect of noradrenaline on HCO_3^- transport occurred both in the antrum and in the fundus This very probably excludes the possibility that the decrease in fundic alkalinization reflected some increase in the (Metiamide inhibited) H^+ secretion

It was however of interest to study the effect of the catecholamines also in fundic mucosa with spontaneous H^+ secretion With noradrenaline and adrenaline (10^{-3}M) (n = 4 for both) there was a slight rise in net H^+ secretion, this probably reflected inhibition of the simultaneous but smaller and therefore masked transport of HCO_3

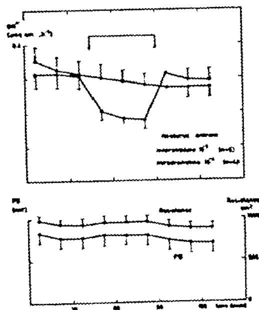


Fig. 3. Noradrenaline (circles) but not isoprenaline (dots) inhibited HCO_3 secretion by *Necturus* antrum. Means ± SE are given. The catecholamine was present in the nutrient solution as indicated. Isoprenaline (10^{-3} M) slightly increased the PD and electric resistance. No changes in these parameters (not shown) occurred with noradrenaline (10^{-4} M).

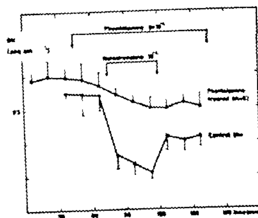


Fig. 4. Pre treatment with phentolamine (5×10^{-4} M) prevented inhibition of the HCO_3 secretion by noradrenaline (10^{-4} M). Fundic mucosae from *Rana temporaria* were used. Only noradrenaline were used in the control experiments (circles). Means ± SE are given.

Isoprenaline however decreased the net H^+ secretion but only at 10^{-3} M ($0.02 < p < 0.05$, n = 6); in a concentration of 10^{-4} M it had no significant effect.

DISCUSSION

An increase in the nutrient Ca^{++} stimulated alkalinization both in the antrum and in the fundus The increased alkalinization was associated with a significant increase in the transepithelial electric potential difference but there was no significant change in the electric resistance this may possibly indicate that HCO_3^- transport stimulated by Ca^{++} is electrogenic Stimulation of gastric HCO_3^- secretion by (intravenous) Ca^{++} occurs also in the guinea pig in vivo (Flemström and Garner unpublished)

Previous work has suggested that cGMP is the second messenger in stimulation of gastric HCO_3^- transport by cholinergic agents and it seems highly probable that this transport originates from the antral and fundic surface epithelial cells (3) Cholinergic stimuli are mediated by cGMP in some other types of cells in the gastrointestinal tract (5 6) and it may be of interest in this context that Ca^{++} is involved in the stimulatory process in these cells

It is well known that cAMP serves as second messenger in stimulation of the H^+ secreting (parietal) cells by histamine but neither dibutyryl cAMP nor histamine affected gastric transport of HCO_3^- (3) Gastrin (penta-gastrin) stimulates H^+ secretion and this very probably occurs by release of histamine (8) This hormone also had no effect on the HCO_3^- transport (Table I) The stimulatory pathway for HCO_3^- transport which probably involves cholinergic stimuli cGMP and Ca^{++} is thus different from the main pathway for H^+ secretion involving cholinergic stimuli gastrin histamine cAMP (and Ca^{++})

Fromm and co workers (9) observed that noradrenaline decreased HCO_3^- secretion in the rabbit antrum The present results indicate that inhibition of HCO_3 transport by noradrenaline occurs both in the antrum and in the fundus and that this effect is mediated by adrenergic alpha receptors Inhibition of HCO_3 transport by adrenergic alpha agonists have been demonstrated also in the intestine (10) It thus occurs in the lumen facing cells in several parts of the gastrointestinal tract

Neither noradrenaline nor adrenaline affected gastric H^+ secretion but as reported before (11) there was some inhibition of H^+ secretion with the beta receptor agonist isoprenaline It was necessary however to use 10^{-3} M of the beta agonist to get an effect on H^+ secretion while the alpha agonist (noradrenaline) inhibited HCO_3 secretion at a concen

tration of 10^{-5} M This may indicate that sympathetic activation of the gastric epithelium primarily affects the secretion of HCO_3

Possible role of gastric HCO_3 transport

It has been suggested that the HCO_3^- transport protects the mucosa from damage by intraluminal acid (1 2 3) and it is likely that due to the higher mobility and usual excess of the H^+ ion neutralization of H^+ by HCO_3^- occurs at the luminal surface of the gastric epithelium The protective ability of the HCO_3^- transport should increase if neutraliza tion occurred in a surface boundary zone with low turbulence rather than in the luminal bulk solution (Fig 5) It may be of interest that the visco-elastic mucous-gel adjacent to the luminal surface of the epithelium seems to have properties (12) appropriate to support HCO_3^- and H^+ gradients The concentration profiles within the surface boundary should depend on the amounts of HCO_3 and H^+ available for diffusion the structure and charge of this zone and on the presence of other ions (cf 13).

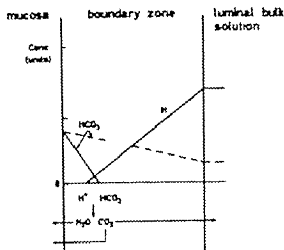


Fig. 5 Model for neutralization of H^+ by HCO_3^- in a boundary zone at the luminal surface of the gastric epithelium With acid gastric conten ts (solid lines) all HCO_3^- in the luminal bulk solution should appear in the form of CO_2 At high rates of H^+ secretion some of the CO_2 may be reabsorbed by the mucosa for utilization by the parietal cells in the H^+ secretory process Free HCO_3^- should appear only in non-acid bulk so lutions (dashed line)

CO_2 and water should be released when H^+ is neutralized by HCO_3 and in guinea pig stomachs with acid contents and low rates of H^+ secre

tion (4) there was good quantitative agreement between loss of H^+ and release of CO_2 Free HCO_3^- appeared only in gastric contents with pH values above 4 but the total amount of HCO_3^- (free HCO_3^- plus CO_2) transported into the gastric lumen was independent of the acidity of the contents At high rates of H^+ secretion however there seems to be some absorption of CO_2 by the mucosa for utilization by the parietal cells in the H^+ secretory process Excess water may diffuse along its chemical gradient ito the mucosa (14) and Na^+ may diffuse into the gastric lumen to maintain electroneutrality when H^+ is neutralized

HCO_3^- in regulation of gastric acidity

The rate of spontaneous (unstimulated) HCO_3^- secretion amounts to 5 - 10 % of the maximal H^+ secretion both in vitro (3) and in vivo (4) This rate of HCO_3^- transport is in quantitative agreement with the well known (15 16) continuous loss of H^+ ions from the lumen of the normal stomach

Gastric HCO_3^- transport stimulated by carbachol dibutyryl-cGMP or Ca^{++} amounts to 10 - 25 % of the maximal H^+ secretion Even stimulated active HCO_3^- transport thus seems insufficient to account for the very high rates of H^+ loss occurring after gastric intraluminal instillation of unionized acetylsalicylic acid and some other agents (cf 17) However under these circumstances there is also increased gastric transepithelial transport of large protein (18 19) and saccharide (20 21) molecules Very high rates of gastric H^+ loss may thus reflect efflux of luminal H^+ or ultrafiltration of interstitial HCO_3 (18 22) through an abnormally permeable epithelium It has also been observed (23) that acetylsalicylic acid which inhibits active gastric transport of HCO_3 at a high concentration increases leakage of this ion through the mucosa

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Effects of Sulfhydryl Reactive Agents on Ion Transport by Isolated Gastric Mucosa

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ABSTRACT The ffects of p-chloromercuribenzene sulfonic acid (pCMBS) wer measured on ion transport by isolated fundic and antral mucosa of th rabbit Fundic mucosa exposed t pCMBS exhibits inhibition of cid secretion which is resistant to stimulation by histamine dibutyryl cyclic AMP and theophylline pCMBS decreases the short-circuit current and increases the tissue electrical resistanc of both fundus and antrum pCMBS increases luminal acid los by fundus but decreases luminal acid l ss by antrum. However pCMBS does not increase th passive unidirectional fluxes of Na and Cl acros fundus or antrum This suggests that the pparent increase in luminal cid loss by fundus exposed to pCMBS is due to the inhibition of acid secretion Th effects f pCMBS a reversed by dithiothr itol (DTT) and cysteine DTT alone but not cy t ine stimulates acid secretion which most likely is an effect of DTT on thiol groups not affected by pCMBS Neither DTT no cysteine increase mucosal permeability t id Th data suggest that oxidation f thi l groups sensitive to pCMBS esults in inhibition of tive ion transport processes without increasing mucosal permeability The thiol reducing agents DTT and cy t in also do not appear t cause an increas in gastric mucosal permeability

F rmation f me captides in th gastri mucosa generally results in the inhibition of acid ecr tion and an increase in mucosal permeability both *in vivo* and *in vitro* However this permeability effect *in vitro* is related to th particular sulfhydryl rea tive agent used (1) Varying degrees f specificity and/o membrane permeation p bably account for some f the differing ffect of these gents The chloromercuribenzene compounds have been used in many tudies because they have a strong affinity f r protein sulfhydryl groups (2) and re believed to lowly penetrate membranes (3) Much of the data on the effects of chloromercuribenzene compound on acid ecr tion come from *in vitro* tudies (1 4-6) and th effects on permeability come from *in vivo* tudies (7 8) One interpr tation f these data is that the ability of th chloromercuribenzene compounds to

addition of pCMBS In contrast to just washing out pCMBS addition of cysteine 4 mM reverses the inhibition of acid secretion caused by pCMBS Table 1 The acid secretory values after addition of cysteine are not significantly different ($p>0\ 1$) from those observed prior to exposure to pCMBS Cysteine alone added to the luminal side f fundic mucosa has no significant effect on the spontaneous rate of acid secretion ($p>0\ 1$) Table 1 Alanine 4 mM which is similar to cysteine except that it does not contain a thiol group does not significantly ($p>0\ 4$) alter the rate of acid secretion by tissues treated with pCMBS Table 1

Histamine 0 09 mM theophylline 10 mM and N^6 O^2 -dibutyryl adenosine 3 :5 monophosphate (DB-AMP) 1 mM added to the serosal side of isolated fundic mucosa of the rabbit have been previously shown to stimulate acid secretion (10 11) However in the presence of pCMBS histamine ($p>0\ 2$) or theophylline ($p>0\ 2$) or DB-AMP ($p>0\ 2$) do not significantly stimulate acid secretion Table 1 In a separate group of experiments each of thes stimulants of acid s cretion wa added to the tissues before th addition of pCMBS Subsequent addition of pCMBS inhibits histamine ($p<0\ 005$) theophylline ($p<0\ 001$) and DB-AMP ($p<0\ 001$) stimulated cid secr tion Table 1 Subsequent addition of another 0 5 mM of pCMBS decreased acid secretion even further (to <0 5 µEq/hr/cm^2) in some (N=7) but not all of the tissues exposed to the above stimulants In 10 tissues initially treated with pCMBS this agent was washed out after acid secretion declined and the acid secretory rate was allowed to stabilize Subsequent addition of theophylline (N 5) or histamine (N=4) stimulated acid secretion by 1 00±0 36 µEq/hr/cm^2 (combined result $p<0\ 05$)

Dithiothreitol (DTT) 2 mM added to the mucosal side of fundic mucosa increase the rate of basal acid secretion by 41% ($p<0\ 001$) Figure 2A. CO_2 measurements indicated that this increase in acid secretory rate was due to an increase in mineral (ie HCl) rather than organic acid production Prior to the addition of DTT the diffe ence between the rates of mucosal and serosal CO_2 appe rance was 2 2±0 6 µM/hr/cm^2 which then incre sed to 3 3±0 6 µM/hr/cm^2 after addition of DTT (N=8) This 50% increase in difference between CO_2 appearanc rates was statistically signi ficant ($p<0\ 025$) In contrast to DTT threitol 2 mM alone added to the mucosal side of 5 tissues had no signifi ant effect ($p>0\ 4$) on the rate of acid secretion

When tissues are pretr ated with DTT which increase the rate of acid

ecr tion pCMBS does not inhibit the acid secretory rat Figure 2B Furthermor when tissues a e pr treated with pCMBS which decreases the rate of acid secr tion subsequent addition of DTT increases the acid secretory rate Figure 2C DTT alone does not ignificantly alter th hort-circuit current r tissue electrical resistance Figure 2D However pCMBS alon decreases the short-circuit current and increases the tissue electrical resistance The mean increase in short-circuit current and tissue electrical resistance after addition f DTT shown in Figur 2E are not statistically significant and the subsequent ddi tion of pCMBS does not ignificantly alter the current resistance Figur 2E When fundic mucosa is initially exposed to pCMBS and then DTT the latter ignificantly increases the short-circuit current (p<0 001) and decreases the tissue electrical resistance (p<0 05) indicating a reversal of the effects of pCMBS Figure 2F

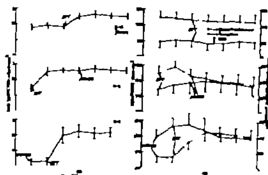


Fig 2 Effects of mucosal addition of DTT 2 0 mM and pCMBS 0 5 mM on acid secr tory rate (A–C) short circuit current and tissue electrical resistance (D–F) See text

In th absence f pCMBS the rates f luminal acid loss by fundus and antrum well as their hort-ci cuit currents and electrical esistances do not change ignificantly over a 70 minute interval once teady-stat conditions ar achieved Howeve in the presenc of pCMBS the rat of luminal cid loss by fundi mucosa increases ignificantly (p<0 001) Figur 3 This change is associated with an 18% increase in tissue lectrical esistanc (p<0 001) and a similar decrease in short i cuit current as bserved t higher luminal pH In contrast t its effect on fundic mucosa pCMBS decreases (p<0 001) rather than increas es the ate f luminal acid loss by antral mucosa Figure 3 pCMBS lso increases the tissue electrical esistance of antral mucosa by 32% (p<0 001) (Figur 3) and decreases the bort-circuit current by 56% (from 54 2±3 0 to 24 1±

Binding of gastric mucosal thiol groups *in vivo* has been implicated as being associated with an increase in mucosal permeability Canine studies have shown an increase in luminal acid loss and luminal Na^+ gain after treatment with pCHBS However the luminal appearance of methylene blue injected intravenously (8) and increased plasma shedding (7) have not been found to accompany the reported increase in membrane p rmeability caused by pCHBS The present results indicate that pCHBS does not increase the permeability of either fundic or antral mucosa under isolated conditions Even though pCHBS increases luminal acid loss by fundic mucos this was not associated with a decrease in tissue electrical resistanc or an increase in the unidirectional flux of another cation Na^+ or anion, Cl^- These data in addition to the observation that pCHBS decreased luminal acid loss by antr l mucosa suggest that the apparent increase in acid permeability by fundus was due to inhibition of acid secretion Another agent used in the present study DTT also has been reported to increase canine mucosal permeability to acid Na^+ and plasma (7 9) The observations that DTT alone doe not significantly affect luminal acid loss by antral mucosa or ignificantly alter the electrical resistance of either antral or fundic mucosa imply that DTT does not increase the permeability of isolated ga tric mucosa Cyst ine which is similar to DTT also does not increase gastric mucosal permeability The effects of pCHBS DTT and cysteine in th present study are compatible with the concept that neither reduction nor oxidation or thiol groups normally accessible to thes agents under isolated conditions result in an increase in mucosal permeability

The differing ffects of pCHBS on gastric mucosal permeability *in vitro* and *in vivo* cannot be r adily explained An alternativ to the usual explana ion of speci or *in vitro*-*in vivo* difference is that the apparent increase in mucosal perme bility reported to occur *in vivo* (7 8) may have been du to i hibition of activ ion transport Inhibition of cid secretion could ause an apparent incr e in luminal loss of acid and inhibition of activ Na^+ ab rption (which occurs in dogs but not rabbits) could result in an ppar nt incr ase in luminal ppearanc of Na^+ Such changes might give ri e t what has been interpr ted as pCHBS causing mild damage to the mucosa *in vivo* It i lso po ible that systemic absorption of pCHBS (19) might indi tly ff t the mu os *in ivo* by r l sing humoral gents from other r (20) Another indir ct me hanism whi h can

affect permeability is r lated to blood flow Fo exampl morphologic data suggest that the increase in permeability in vivo caused by DTT is the result of tasis and distention of mucosal capillaries (21) which is consi tent with the present data suggesting that DTT does not increase gastric mucosal permeability in vitro

TABLE 1 Effects of pCMBS on Rates of Acid Secretion (μEq/hr/cm^2)

N			
10	basal 1 97±0 31	pCMBS 0 17±0 12	pCMBS reversed 0 43±0 20
10	basal 1 96±0 33	pCMBS 0 41±0 25	cyst ine 1 58±0 43
10	basal 2 12±0 26	cyst ine 2 20±0 24	–
5	basal 1 86±0 30	pCMBS 0 20±0 18	alanine 0 21±0 20
10	pCMBS 0 36±0 10	histamine 0 43±0 15	–
5	pCMBS 0 30±0 08	theophyllin 0 52±0 13	–
5	pCMBS 0 40±0 13	DB-AMP 0 60±0 21	–
5	histamine 3 35±0 50	pCMBS 0 71±0 30	–
5	theophylline 4 33±0 55	pCMBS 1 25±0 35	–
5	DB-AMP 3 87±0 47	pCMBS 0 99±0 30	–

Values are given as mean ±1 SEM. pCMBS 0 5 mM, cysteine 4 mM, and alanine 4 mM wer added to the mucosal id Histamine 0 09 mM as ba theophylline 10 mM and DB-AMP 1 mM ve added to the erosal id

TABLE 2 Effects f pCMBS on Unidirectional Mucosal to-Serosal Fluxes (J_{ms}) f Na^+ and Cl^-

N	Agent Added	FUNDUS J_{ms}^{Na}	FUNDUS J_{ms}^{Cl}	ANTRUM J_{ms}^{Na}	ANTRUM J_{ms}^{Cl}
10	–	2 8	4 1	2 5	3 8
		3 1	4 3	2 7	3 7
Δ		0 3±0 3	0 2±0 3	0 2±0 1	–0 1±0 1
10	–	3 0	4 0	2 4	3 4
	pCMBS	2 7	3 6	2 1	3 2
Δ		–0 3±0 2	–0 4±0 4	–0 3±0 2	–0 2±0 1

J_{ms} x μEq/hr/cm^2 Δ = mean ±1 SEM diff rence between the two measurements

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Mechanisms of Action of Aspirin on the Gastric Mucosa of the Guinea Pig

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ABSTRACT: Autoradiography was used to determine distribution f ^{14}C-aspirin in sections from the stomach wall of the guinea pig Immediately after intragastric instillation of 20 mM (3 6 mg ml $^{-1}$) for 10 min at pH 2.0 grain density in the upper region of the glandular mucosa was 15-fold greater than in the submucosa and the salicylate concentration in the stomach wall was 1 28 mg g^{-1} Within 30 min mucosal grain density had fallen to the level in the submucosa and gastric salicylate concentration was similar to that in th blood (0.03 mg g^{-1}) There was a higher grain density in the mucosa compared with the submucosa after instillation of 40 mM aspirin for 10 min at pH 7 4 However gastric salicylate was less than at pH 2 0 (0 94 mg g^{-1}) and the drug was found to accumulate in the extracellular compartment f the mucosa. It is likely that the high intracellular salicylat concentration attained following oral administration of aspirin in the nonionised form is responsible for initiating gastric mucosal damage Secondary factors including inhibi tion of gastric alkalin secretion and an increas in the rate of mucosal cell loss seem to be involved in the full development of lesions

It is well known that aspirin damages the gastri mucosa following oral administration in the nonionised form (1 2) However the underlying mechanism responsible for an increase in mucosal permeability and formation of haemorrhagic erosions is unknown A widely discussed theoretical model proposes that gastric toxicity is due primarily to accumulation of drug anions in the surface mucosal cell Intracellular accumulation of weak acids in plant and animal tissues was originally postulated by Overton (3) and later with specifi reference to aspirin-induced gastric damage by Martin (4) In the normally acidic gastric lumen aspirin is predominantly nonionised and therefore rapidly absorbed but on entering the neutral environment of the mucosal cells the drug becomes ionised and is trapped It is possible that dissociation of the acid may disturb the internal osmotic stability and buffer system of the

cell while the high concentration of anions may interfere with intra cellular metabolism.

A number of indirect observations which support this hypothesis have been reported (5 6 7) In the present study distribution and concentration of radiolabelled aspirin in the stomach wall of the guinea pig were investigated directly using an autoradiographic technique Labelled material was immobilized by freezing and the sections exposed in contact with stripping film at −30 C in order to prevent translocation of the drug Since pH has a marked influence on the degree of damage (8 9) aspirin was administered in both the nonionized and ionized forms

Methods

Experiments were performed on male albino guinea pigs (470–530 g) which were starved for 24 h before use but allowed free access to drinking water Surgical procedures and the method for filling and emptying the stomach have been described previously (10) Carbon-14 carboxyl labelled aspirin (^{14}C aspirin) with a specific activity of 0 25 µCi mg $^{-1}$ was administered in a dose volume of 10 ml and removed from the stomach after 10 min The drug was administered in concentrations of 20 mM at pH 2.0 (isotonic NaCl containing 10 mM HCl) and 40 mM at pH 7 4 (isotonic phosphate buffer) At other times the stomach was filled with 10 ml of isotonic NaCl Body temperature was maintained at 35 C

Animals were sacrificed immediately after removal of the drug (time 0) and 10 30 and 60 min later The stomach was removed as rapidly as possible and placed in a freezing mixture of cyclohexane cooled with solid carbon dioxide Tissue blocks were prepared from the body region and stored at 30 C At the time of sacrifice a blood sample (~5 ml) was obtained by cardiac puncture and stored in a heparinized tube

The technique of preparing autoradiographs was adapted from that developed for autoradiography of soluble labelled compounds (11) Under red safe light illumination microscope slides were covered with pieces of stripping film (Kodak AR10) with the emulsion upwards Transverse sections (6–8 µm) were cut in the darkroom at 30 C with a Pearse cryostat and placed in contact with the film The slides were placed in light tight boxes and stored for 7 days at 30 C At the end of the exposure pe iod lid were allowed to reach room temperature then dipped in 1% w/ gelatin to prevent movement of the sections during subsequent t tment and dried in a current of warm air Tissues were

fixed in absolute methanol for 15 min then washed in several changes of distilled water Autoradiographs were developed for 4 min at 21 C (Kodak D19b) rinsed in distilled water for 5 min fixed for 5 min at 21°C (Kodak F-5) then washed in running tap water for 10 min. The sections were stained in azure and eosin for 1 1½ hr dehydrated in isopropyl alcohol (50% up to absolute) and cleared in xylene

Autoradiographs were examined by light microscopy (x500) and the grain density determined with an eye piece graticule (400 μm^2) Grains were counted over the mucosal and submucosal regions in each section and subtracted from the background count At higher magnification (x1250) the mucosal region was systematically scanned and the percentage of grains occurring over cells or extracellular space recorded. A locating graticule was used to prevent error during refocusing since the grains and section were not in the same focal plane at this magnification The percentage volume fraction f these two compartments was determined stereoscopically

Absorption of aspirin was determined from the decrease in concentration of the administered solution following intragastric instillation for 10 min Drug concentrations were determined as total salicylate by liquid scintillation counting (Packard 3003 TriCarb) and corrected for quenching with an external barium standard. Total salicylate concentrations in the stomach wall and the blood were determined by tissue combustion (Packard 306 TriCarb sample oxidiser) and the efficiency of counting calculated by the internal quench method In the case of the stomach wall approximately 20 sections (10-20 mg) were cut from each tissue block, placed in a preweighed paper planchette and th accurate weight of the tissue recorded A blank was included in each batch of samples to obtain the background count

The influence of oral administration of aspirin on the rate of exfoliation f gastric mucosal cells in the guinea pig was determined by measurement of the DNA concentration in gastric washings (10) Total acid and alkaline gastric secretions were determined by measurement f intragastric pH and pCO_2 (12) and the effect of aspirin studied following intravenous administration

Results

Immediately following removal of a solution of 20 mM aspirin at pH 2.0 from the tomach labelled material was present throughout the upper

(luminal) region of the glandular mucosa Within the mucosa there was a sharp demarkation indicating the depth to which the drug had permeated although grains were fairly evenly distributed within this zone (Fig 1) A marked reduction in mucosal grain density was observed in stomachs fixed 10 min later but a high concentration of grains was still present over the superficial region of the mucosa adjacent to the luminal surface (Fig 2)



Fig 1



Fig 2

Fig 1 Autoradiograph of the gastric mucosa from the body region of the stomach The tissue was fixed immediately after (time 0) intragastric instillation of 20 mM ^{14}C aspirin for 10 min at pH 2 0 The lumen is at the top of the photograph

Fig 2 Autoradiograph of the stomach wall from the body region of the stomach The tissue was fixed 10 min after exposure of the mucosa to ^{14}C aspirin at pH 2 0

Grain density in the mucosal region adjacent to the luminal surface and in the submucosa at various times after instillation of 20 mM ^{14}C-aspirin for 10 min at pH 2.0 is shown in Fig 3 Immediately after removal of the drug mucosal grain density was 15 times greater than in the submucosal region There was a subsequent fall in mucosal grain density which reached a value similar to that in the submucosa after 30 min Aspirin concentrations in the stomach wall and the blood were

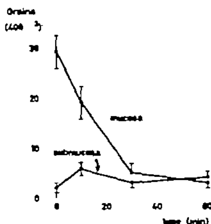


Fig 3

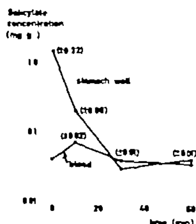


Fig 4

Fig 3 Grain density in th mucosa and submucosa following intragastric instillation of 20 mM ^{14}C aspirin for 10 min at pH 2 0 Means ± SE are shown n = 8

Fig 4 Total salicylate concentrations in the stomach wall and the blood following intragastric instillation of 20 mM ^{14}C aspirin for 10 min at pH 2.0 Means ± SE are shown, n = 8

determined as total salicylate and are shown in Fig 4 The salicylate concentration in the stomach wall (1 28 mg g^{-1}) was approximately 40-times greater than that in the blood immediately after administration Most of the drug was however confined to the upper region of the mucosa (see Fig 1) and thus the concentration in the actual mucosa is likely to b considerably greater than the value measured

There was also a high grain density over the mucosal region of the tomach after instillation of 40 mM ^{14}C-aspirin for 10 min at pH 7 4 (Fig) In stomachs fixed 10 min later autoradiographs revealed a fairly even distribution of grains throughout the glandular mucosa as opposed to the high grain density which remained over the surface mucosal cells after administration of aspirin in the non-ionized form (see Fig 2)

The aspirin treatments used (20 mM at pH 2.0 and 40 mM at pH 7 4)both damaged the stomach as evidenced by an increase in the rate of mucosal cell los d termined from the DNA concentration in the gastric washings (Fig 6) However damage was considerably less when the drug was



Fig 5 Autoradiograph of the gastric mucosa from the body region of the stomach The tissue was fixed immediately after (time 0) intragastric instillation of 40 mM ^{14}C-aspirin for 10 min at pH 7 4 The lumen is at the top of the photograph

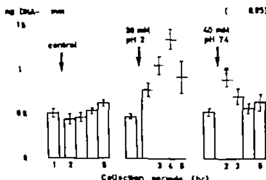


Fig 6 Rates of accumulation of DNA (mean $\pm$ SE) in guinea pig gastric washings before (1st h) and after (2nd–5th h) intragastric instillation of aspirin The dose volume was 10 ml and the drug solution was present in the stomach for 10 min Asterisks denote rates of DNA accumulation significantly greater ($p < 0.05$) than those before aspirin administration At pH 2.0 n=10; at pH 7 4 n=8

administered in the ionised form Some of the factors which may contribute to this reduction are shown in Table 1 Gastri absorption and the salicylate concentration in the stomach wall confirmed by grain density measurement were lower when aspirin was administered in the ionised form at pH 7 4 In addition the drug was found to accumulate in the extracellular compartment of the mucosa. A ratio (extracellular grains/extracellular volume) of unity is indicative of an equal drug distribution b tween the cellular and extracellular compartments This was approximately the case when aspirin was administered at pH 2 0 (ratio = 1 09) but when the drug was given in the ionised form at pH 7 4 the ratio was 1 45

The appearance of aspirin-induced gastri erosions in the guinea pig 3-4 h after dosing (13) contrasts markedly with the presence of a high salicylate concentration in the mucosal cell A possible explanation

TABLE 1 Absorption concentration and mucosal distribution of salicylate in the guinea pig stomach following intragastric aspirin The drug was administered at a concentration of 20 mM at pH 2 0 and 40 mM at pH 7 4 Mean values ± SE are given n = 8 for both groups

pH	Absorption (%)	Concentration (mg g^{-1})	Grains (400 u^{-2})	Extracell grains(%)	Extracell volume(%)
2 0	31 1 ± 0 8	1 28 ± 0 22	29 ± 4	12 ± 2	11 0 ± 1 4
7 4	18 1 ± 0 8	0 94 ± 0 35	21 ± 6	17 ± 3	11 7 ± 2 1

for this difference in time scales is the involvement of secondary factors in the full development of lesions Gastric alkaline secretion may be important since this aspect of mucosal protection is particularly sensitive to inhibition by aspirin Basal output of HCO_3^- was reduced following intravenous administration of 5 mg kg^{-1} aspirin (Fig 7); the dose of aspirin used in most other phases of this study (20 mM) is equivalent to 180 mg kg^{-1} At a higher dose of 100 mg kg^{-1} aspirin caused a loss of H^+ ions and a gain in luminal HCO_3 and Na^+ ions (Fig 8), characteristic of an increase in mucosal permeability In thes net

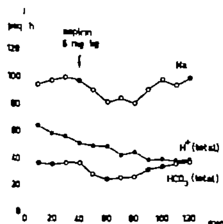


Fig 7

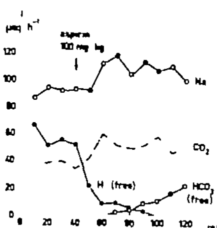


Fig 8

Fig 7 Effect of intravenous injection of 5 mg kg^{-1} aspirin on gastric outputs of H^+ HCO_3 and Na^+ ions in the guinea pig At this dose level the drug produced a decrease in alkaline secretion

Fig 8 Effect of intravenous injection of 100 mg kg^{-1} aspirin on gastric output of H^+, HCO_3 and Na^+ ions in the guinea pig At the higher dose level, the drug caused a loss of luminal H^+ and a gain of HCO_3^- and Na^+ ions

acid secreting preparations all HCO_3 was converted to CO_2 and there was a decrease in gastric CO_2 formation after 5 mg kg^{-1} aspirin whereas in the presence of an increase in mucosal permeability there was a rise in CO_2 output

Discussion

Salicylate distribution in the gastric mucosa has been the subject of considerable discussion in relation to the mechanism of aspirin-induced damage but has not been investigated experimentally Morris and co-workers (14 15) have related gastri salicylate concentration to mucosal damage in the rat after oral administration of ^{14}C aspirin The same group also studied absorption and distribution of the drug by placing X ray plates in contact with the mucosal and serosal surfaces of the stomach This approach however provides only limited information on mucosal distribution which requires study in the opposite (transverse) plane of the stomach wall

At pH 2 0 direct examination of the autoradiographs revealed that the drug accumulated in the outer mucosal region At this time the concentration in the stomach wall was about 40-times greater than in the blood A high level of salicylate remained in the surface layer of epithelial cells 10 min after removal of aspirin from the lumen but within 30 min the drug was evenly distributed throughout the stomach wall and the concentration was similar to that in the blood

An obvious discrepancy between the observed data and the formation of gastric lesions concerns the even distribution of the drug over the whole of the upper mucosal region whereas erosions are characteristically focal. In addition the presence of a high mucosal salicylate concentration and the appearance of gastric erosions represent vastly different time scales. For this reason it is possibl that the dwell time of salicylate in a particular cell is important in determining the ite of damage After oral administration of aspirin in the nonionized form the drug was present in the surface mucosal cells for the longest time period which corresponds to the region of damage e g the site of increased permeability to tissue electrolytes and macromolecules (1 16) and the site of gastric cell loss (7 10) The difference in time course between salicylate accumulation and lesion formation may be due to the involvement f secondary factors including inhibition f gastric alkaline ecretion which normally protects the mucosa from damage (17)

and back-diffusion of H^+ ions (1) Gastric HCO_3^- secretion was reduced by low concentrations of aspirin administered from the blood side and therefore in the ionized form indicating that this aspect of mucosal protection is particularly sensitive to the drug In the isolated gastric mucosa inhibition of alkalinization has been demonstrated as a common property among a variety of ulcerogenic agents such as acetazolamide (17) aspirin (18) fenclofenac parathyroid hormone and noradrenaline (Flemstrom personal communication) Conversely the possibility that specific stimulants of alkalinization may protect the mucosa has implications in the development of a new approach in the treatment of gastric ulceration

Although there was a high grain density in the outer mucosal region following oral administration f aspirin at pH 7 4 gastric absorption and mucosal concentration were les than at pH 2.0 In addition the drug was found to accumulate in the extracellular compartment i e entry of salicylate into the mucosal cells was inhibited which may further contribute to the reduction in damage It is possible that absorption of ionised aspirin from th stomach i facilitated by a low pH at the absorptive surface *as a result of acid secretion However under such* circumstances a similar distribution pattern would occur irrespective of pH. The fact that different distribution patterns were found at the two pH values could be interpreted in terms of an extracellular route of absorption of the charged form of a weak acid as suggested previously(5).

It seems likely that the high mucosal concentration of salicylate attained after oral administration of aspirin in the nonionised form is responsible for initiating gastric damage The fact that many of the mucosal cells are apparently resistant may be related to the dwell time of the drug in particular cells It is also possible that many cells sustain moderate damage which is reversible on disappearance of salicylate from the mucosa The high rate of turnover of the gastric epithelium may also b significant in providing some degree f protection.

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The Transient Nature of Equivalent Circuits, in the Sea, on the Land On the Meaning of Epithelial Conductance

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For this symposium honoring Professor Torsten Teorell it is fitting that attention be drawn to his pioneer study of the impedance characteristics of the isolated gastric mucosa (1) Of equal value for one considering the transient behavior of trans-epithelial potential differences is his critical analysis of artificial membranes (2) His work led to an admonition (3) that needs to be restated some thirty years later

> The exact significance of the parameters in the GILDEMEISTER LULLIES-COLE method of characterizing cell or tissue impedance is far from clear Hence great caution is necessary in all attempts to interpret these impedance quantities in terms of cell or tissue permeability

A few years later Professor Teorell s work was a colloquium topic at the National Heart Institute Dr K.S Cole suggested that the electrical transient of the gastric mucosa be compared with that of an artificial membrane developed by Dr Karl Sollner (4) Exchange-diffusion of chloride through the gastric mucosa had been identified in the laboratory of Professor Hans H Ussing as a discrepancy between a unidirectional flux of chloride and the mucosal steady state conductance(5) The oscilloscopic images of the transients developed by the bullfrog gastric mucosa and by a Sollner-membrane were for practical purposes superimposable Thus it is not surprising that the memory is vivid today as the picture of 25 years ago The association of Dr Gottlieb with the laboratories culminated in a study of flux and conductance in artificial membranes (6) The latter observations have been replicated and perhaps a level of sophistication that may have prevailed in 1974 is reflected by a quotation (with emphasis added)

> the electrical resistance was calculated from Ohm s law <u>transiently</u> setting ΔV at ±10 mV and recording <u>steady state</u> values of the electrical current (7)

In recent years the parameter conductance has become more than an exchange diffusion esoterica For many concerned with agents injurious to the stomach as reflected by several contributions to this Symposium mucosal conductance may well be the dominant parameter of interest

Convergence of two circumstances an ongoing comparative study of the electrophysiology of the gastric mucosa and the acquisition of a 230 gram programmable calculator has prompted reevaluation of epithelial conductance

Consider a mucosa positioned for electrophysiological study between a pair of proximate voltage (PD) sensing electrodes and a pair of more distant current-delivering electrodes Upon sending a constant current ΔI through the mucosa the transmural potential difference abruptly or instantaneously' goes to a new value After an interval of milliseconds seconds or even minutes the potential difference reaches a new steady state This progression or the voltage transient has been found experimentally to fit the equation

$$\pm PD/\Delta I \quad a_0 + a_1(1-e^{k_1 t}) + a_2(1 \; e^{k_2 t}) + \\ a_n(1-e^{k_n t}) \qquad (1)$$

where t time and the sign is determined by the polarity of ΔI The same constants appear in the counterpart equation for the time-dependent change of current when the PD is displaced by a voltage-clamp operated in the 'OFF' mode Plotted semi logarithmically the transient can be resolved into several straight lines each with an intercept a having dimensions of $\Omega.cm^2$ and a slope that is a rate constant k the reciprocal of which will be given here as a half time $t_{½}$ Perhaps the most remarkable feature of a transient is that it appears to resolve into very few two to four exponential terms As equation (1) stipulates the values for a and k are within reason (e g a displacement of the spontaneous potential by less than 75 to 100 mv) independent of the polarity or magnitude of the current pulse

We shall refer to the ideal linear symmetrical transient of an epithelium as the *pauci-exponential transient* (Some epithelia display an overshoot or an inductance component (2) which will not be considered in the present context. The transient of the isolated rat gastric mucosa peaks at about 1 minute reaching a level about 10% greater than the steady-state value unpublished observation)

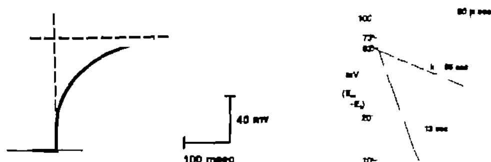


Figure 1 The *pauci-exponential* transient developed by the gastric mucosa of *Squalus acanthius*

Both surfaces of an isolated gastric mucosa were exposed to normal elasmobranch saline (11) A current pulse of 233 μamp/cm displaced the PD to 100 mv mucosal face negative The broken horizontal line conveys a superimposed trace photographed six minutes later (#4 8/5/74)
At the right the curve has been fitted by least squares to two exponential terms and plotted semi logarithmically

Parameters of the transients elicited from gastric mucosae of the leopard frog (8) the spiny dogfish Figure 1 and the rabbit fetus (9) are given in Table 1 For each species both surfaces were bathed by a physiological saline that is appropriate to its vertebrate class For two mucosae that are both functionally and structurally different those of dogfish and the rabbit fetus the transient parameters are qualitatively not very different On the other hand the mucosae of the frog and dogfish which are functionally distinct (10a b) but structurally indistinguishable by light microscopy have transients that are qualitatively dissimilar Though much is uncertain it is likely that transients arise at permselective ionic paths or boundaries Nevertheless substitution of choline for sodium in the solution bathing the mucosal surface of the dogfish and rabbit fetal mucosae did not materially perturb their respective transients

TABLE 1 EXAMPLES OF TRANSIENTS DISPLAYED BY GASTRIC MUCOSAE

COMPONENT	Normal saline at both mucosal and serosal surfaces				Mucosal Serosal	choline normal	saline saline.
	$t_{\frac{1}{2}}$ msec	Intercept Ω cm^2	$t_{\frac{1}{2}}$ msec	Intercept Ω cm^2	$t_{\frac{1}{2}}$ msec		Intercept Ω cm^2
Rana pipiens (8)							
Fastest	1 7 3	91 14					
Fast	12 1	35 6					
Slow			3 3 6	67 12			
Slowest			22 3	166 20			
Non reactive		22 4					
Squalus acanthius							
Fastest	6 2 5	40±4			7 2±1 1		45 5
Fast	33 2	109 12			26 4		115 21
Non reactive		53 9					16 9
Rabbit f n (9)							

ERRATUM

page 114 3rd columne $t_{1/2}$ msec

should read $t_{1/2}$ sec

A study by Dr M.G. Kienzle and myself on the transient of the dogfish gastric mucosa has been summarized in Table 2 A standard or control physiological elasmobranch saline used for 21 seasons has the following composition

	mEq/l		
Na^+	252	Cl	240
K^+	10	HCO_3	30
Ca^{2}	10	SO_4^{2}	4
Mg^{2}	4	PO_4^{2}	2

with 28 mM of glucose and gassed by 5% CO_2 95% O_2 (11) Two test solutions were prepared with 220 mEq/l of Na^{+2} 87% replaced by either 220 mEq/l of choline or THAM(tris[hydroxymethyl]aminomethane) For an additional two solutions 220 mEq/l of Cl 92% were replaced by either isethionate or glucuronate Within the scope of this study the choice of the substituent ion to replace either Na^+ or Cl did not determine the outcome Consequently the respective results have been pooled Reported values for the percent change in the parameters are based on paired differences between the test and control saline solutions

To establish a new ionic environment the bathing solutions were changed twice three minutes apart with the exchange being 99% complete Mucosae *from 6 fish provided* records of 40 transients recorded 16 ±4 (SD) *minutes* after the first change of solutions and an additional 20 records were obtained 7 ±3 minutes later There is no assurance that the lapse of time about twenty three minutes would be sufficient for intracellular ion concentrations to reach an approximate new steady state The interval between the change of solution and recording a transient had to be a compromise between attaining a steady state within the tissue and the recognized changes that occur even in the absence of ion substitution. An apparent "half time' for the complete signal system, pulse generator current delivering electrodes bridges etc. with no mucosa present in the flux chamber was less than 50 µsec (electrical fields in Halsey #1 precluded closer scrutiny)

For the results given in Table 2 we used a relatively large current pulse of 230 µamp cm^2 However the response was linear even after ionic substitution over the range of 23 780 µamp cm^2 By superimposing traces obtained at intervals up to 10 minutes later it was evident that

TABLE 2 IMPACT OF REPLACING SODIUM OR CHLORIDE ON THE TRANSIENT OF THE DOGFISH GASTRIC MUCOSA.

Per cent deviation from values when normal saline was at both surfaces

Bathing Solutions		Fastest Component		Fast Component		Total (DC) Resistance
Serosal surface	Mucosal surface	$t_{½}$	Intercept	$t_{½}$	Intercept	
Na replaced	Normal	15	10	2	9	22
Na replaced	Na replaced	1	+30	15	-3	Too few
Normal	Na replaced	15	31	2	+20	Too few
Cl replaced	Normal	13	+ 9	11	+37 (+29)	48 (+45)
Cl replaced	Cl replaced	19	18	38 (23)	23	69 (28)
Normal	Cl replaced	1	36 (+23)	+ 9 (+ 7)	6	16 (+13)

Eighty seven per cent of the Na^+ replaced by choline or $THAM^+$ and ninety two per cent of the chloride replaced by isethionate or glucuronate

Per cent deviations were within the 95% confidence limits except where a limit is given in parenthesis

there were no demonstrable components in this species whose $t_{½}$ exceeded those given

Clearly the transient parameters are not very dependent upon the ionic milieu While the total DC resistance increased significantly after anionic substitution a relatively trivial observation only two of the half times were modified significantly and for one of these Cl replacement at the mucosal surface the mean difference barely exceeded the 95% confidence limit

An equivalent circuit for a component of a transient can be built from a subcircuit of a resistance and capacitance in parallel For each such pair the time constant is given by the product of the resistance (R) and the capacitance (C) so that

$$t_{½} = 0\ 69\ RC \tag{2}$$

It is conventional wisdom that those components having a half time in the millisecond range arise from 'true' or dielectric capacitances attributable to plasma membranes Thus for the fast transients developed by the dogfish we might not expect C in equation (2) to be especially susceptible to ionic substitution But if the initial assertion were accepted it would be somewhat surprising if R did not increase and thus lengthen one or both half times



Figure 2 The physiological gastric mucosa of *S acanthias*

A gastric mucosa was stretched but only lightly so as we customarily mount it in a flux chamber At the conclusion of an experiment the mucosa was fixed in 10% formalin The thickness noted here 2 mm is within the span indicated by a wet weight of 220 ±53 (SD) mg/sq cm (10b) obtained earlier (#38 Courtesy of Dr L.B. Longley)

With no attempt at sophistication the structures of a gastric mucosa Figure 2 lead one to expect that the following would generate significant components of a transient

1) An unstirred layer at the mucosal face

2) The apical plasma membranes of at least 2 dominant cell populations (Even non-mammalian mucosae have a potentially significant third cell type the mucous-neck cell (12) in addition to the oxyntic and surface epithelial cells)

3) Cellular-polarization of at least 2 populations

4) Basolateral plasma membranes of the same cells

5) Electrolyte compartments formed by para-cellular shunts

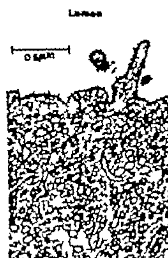


Figure 3(a) Apex of an oxyntic cell

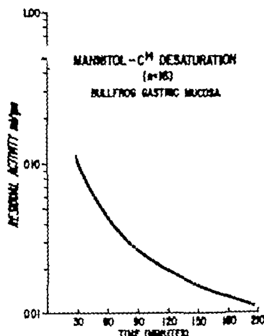


Figure 3(b) Submucosal escape of mannitol

(a) Accentuation of the apical microtubular system in the oxyntic cell of *Squalus acanthius* The tubular system was accentuated by inhibiting acid secretion with 25 mM SCN (#209b 1964 Courtesy of Dr A W Sedar)
(b) The submucosal tissue of the gastric mucosa of *Rana catesbiana* was exposed to labelled mannitol for 4 hours The subsequent desorption of mannitol was arbitrarily fitted to a series 3-compartment model The resultant percentage for extracellular spaces and the corresponding half times became 71% 8 minutes 23% 20 minutes and 6% 1 0 minutes (14)

6) The unstirred layer of the thick submucosal tissue

Thus from this abridged list if separable we could expect the transient to have 10 to 14 components or exponential terms But this is only the tip of the iceberg The mucosal unstirred layer extends into complex gastric tubules and even onto apical microtubules Figure 3(a) (13) The submucosal layer is also complex The washout or desorption of inert extracellular markers from this slab with fingers between tubules follows a multiexponential course (14) Figure 3(b)

Transients have a dearth of exponential terms in spite of an expectation that the number of terms could well be infinite This appears to have been dismissed by some although not by this author on the grounds that many if not most of the terms are insignificant and others are not separable It is this distinctive feature that led to the choice of the qualifier for the pauci-exponential transient

Cellular polarization is the expected occurrence when an epithelial cell has opposite membranes of different ionic conductances One could readily construct a model from sheets of 2 artificial membranes having different permselectivities and separated by a finite volume of electrolyte solution The expected transient behavior is analyzed in Figure 4

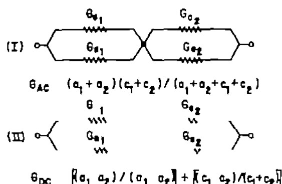


Figure 4 Cellular polarization (15 17)

Conductances are given for a cation c and an anion a at surfaces 1 and 2 The overall conductance following a constant current pulse is given for t=0 G_{AC} and in the steady state by G_{DC} t=∞ In flammatory time- variant e.m.f s entrained in this model have been deliberately omitted Inclusion of an active transport e.m.f is optional but would not contribute to clarity Though not necessarily correct each partial conductance is treated as if it were independent of the electrochemical PD

Since it is reasonable to expect most epithelial cells to have apical and basolateral membranes whose permeabilities differ and often strikingly so 'cellular polarization should account for an important part of a transient and may be the probable origin of very long time constants The cell is an electrolyte compartment displaying conservative properties The transition of the transcellular PD from t=0 to t=∞ due to a current pulse is a transition through an unsteady to a new steady state as the cell accumulates or loses energy There are other electrolyte compartments such as the unstirred layers and paracellular shunts which should react in a comparable fashion They may be less amenable to superficial commentary because their geometric configurations appear to be less simple Collectively these comprise 'polarizable electrolyte-compartments Since the term is felicitous we shall designate these polarizable electrolyte-compartments the *ionic-capacitors* Figure 5

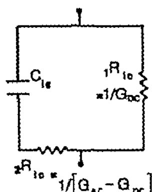


Figure 5 The equivalent circuit of an *ionic capacitor*

This is an equivalent circuit for the elements displayed in Figure 4 A complaint that neighborhood stores do not stock sufficiently large capacitors appropriate for most epithelia while justified should not deflect us from logical perception When values for the partial conductances become known it will be reasonable to replace the capacitor by a time-variant e m f The subcripts for G AC & DC have been carried over from Figure 4 The subscript *ic* will be used for components of an ionic-capacitor The resistance subscripts 1 and 2 identify two resistors

The impetus for developing the designation ionic-capacitor is to take a small step towards relating electrical phenomena to histological structure For those who find equivalent circuits ethereal or even evanescent electrical subcircuits if properly identified are analogous to tracer compartments

If we confine ourselves to 3-compartment arrays we can have the 3-compartments arrayed in series a catenary system of 2 pellucid pools with water from melting snow cascading from the higher to the lower separated from a fjord by a waterfall A marker will follow a transient course and each exponential term will uniquely characterize the volume and decay rate of the corresponding pool It is simple and easy to grasp but its relevance to the real world is dubious

But the three compartments may be in a mammillary array (18) with two higher but separated pools emptying into a lower common pool Histori cally this author became aware of the mathematical solution for the 3-compartment mammillary system (19) in the course of studying ionic flux. In this array the constants of an individual exponential term no longer uniquely characterize a compartment. In the simplest case the tracer exchange is described by a pauci-exponential equation with 3 terms and 2 decay constants Now each apparent rate constant is a mathematical function of both of the two individual rate constants that characterize tracer kinetics between one of the small pool s compartment alone and the large pool

It would be meritorious to periodically relate electrical and compart mental analogues Traditionally biophysical equivalent circuits are two dimensional as are all the figures in this paper It is more generally appreciated in tracer analysis that a real array may have three dimensions (20)

Though the complex systems we are considering epithelia are found experimentally to be linear this does not establish that the constituent parts are also linear

The equivalent circuit for a two dimensional mammillary system is that of 2 RC subcircuits coupled in a parallel fashion Figure 6

Considered either as compartments or RC subcircuits parallel coupling is ubiquitous It is only the most unusual circumstance that even per mits considering a series or catenary array We inhabit a three dimen sional extended mammillary universe

The mathematical solution for two RC subcircuits coupled in parallel is derived for the equivalent circuit of Figure 6

Since the contribution of Rehm and Tarvin to this symposium is acces sible to the reader their notation has been adopted Their coupling

resistors $R_3 + R_4$ have been replaced by two equal resistors whose sum is R_3

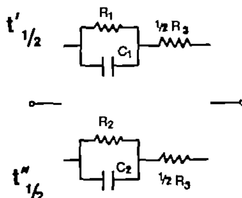


Figure 6 See text

After minor algebraic rearrangement of their equations (8) (11) deemed appropriate for a 50 step calculator we have

$$t_{½} = V/(\theta \quad I\ ) \tag{3}$$

$$t_{½} \quad V/(\theta + I') \tag{4}$$

where $$V = 1\ 4(R_1R_2R_3C_1C_2) \tag{5}$$

$$\theta = R_1C_1(R_2+R_3) + R_2C_2(R_1R+R_3) \tag{6}$$

$$\Gamma \quad ([R_1C_1(R_2+R_3) \quad R_2C_2(R_1+R_3)]^2 + 4\ R_1^2R_2^2C_1C_2)\ ^2 \tag{7}$$

To stimulate a gastric mucosa we have selected the following components

a) a 3µFd di-electric capacitor shunted by a 1200Ω resistor for a plasma membrane

b) an 80 mFd electrolytic capacitor shunted by 560Ω and arbitrarily labeled an 'oxyntic cell"

c) a 1 mFd electrolytic capacitor shunted by 3300Ω and designated perhaps without warrant a 'surface cell

d) two coupling resistors 9 1 and 33Ω to unite two pairs of sub-circuits

The coupled and uncoupled half times were computed as given in Table 3 While the identities of the units giving rise to the half times are fictitious and the values given for the parameters are choices based on

TABLE 3 ILLUSTRATIVE INTERACTIONS BETWEEN CAPACITORS

		$t_{½}$ sec	R Ω	C μFd	Coupled R_3 Ω
The First Interaction					
'Plasma Membrane'	coupled	$6\ 7\ 10^{5}$	1200	3	33
	uncoupled	$2\ 5\ 10^{3}$	1200	3	—
'Oxyntic Cell	coupled	21	560	$8\ 10^{4}$	33
	uncoupled	31	560	$8\ 10^{4}$	—
The Second Interaction					
"Surface Cell	coupled	$6\ 2\ 10^{3}$	3300	$1\ 10^{3}$	9 1
	uncoupled	2 3	3300	$1\ 10^{3}$	—
'Oxyntic Cell	coupled	27	560	$8\ 10^{4}$	9 1
	uncoupled	31	560	$8\ 10^{4}$	—

The reader s indulgence is sought for the pedagogical privilege of arbitrarily specifying the cell types

convenience two qualitative conclusions emerge from parallel coupling which have been designated as the *first and second interactions* As Rehm and Tarvin have derived in this symposium, a short half life may be shortened even further by coupling with a longer half life The *interactions* predict that

A Parallel coupling between a dielectric capacitance and a ionic capacitor may drive the exponential term of the former into the microsecond range or even into the arena of 'noise' *the first interaction*

B Coupling of two ionic capacitors in parallel may lead to the one having a shorter half life being expressed in the millisecond range and susceptible of being mistaken for a dielectric capacitance *the second interaction.*

With respect to the transient developed by the gastric mucosa of the dogfish

1 It is likely that both the relatively short half times of 6 & 33 milliseconds are generated by ionic capacitors Components derived from plasma membranes either did not extend into the milli second range or disappeared as a result of parallel coupling
2 Though tenuous one might entertain the possibility that the absence of a very long half life signifies an oxyntic cell whose opposite membranes are substantially less asymmetric than encountered in other vertebrates since absence of one or more long half times e g 30 seconds is associated with other unique features of the elasmobranch gastric mucosa (10b 21)

ON THE MEANING OF EPITHELIAL CONDUCTANCE

Placing parallel RC subcircuits in a semiquantitative perspective leads to a reconsideration of transmural mucosal or epithelial conductance

In spite of the intricate geometry of the gastric epithelium encountered in Figure 2 for those accustomed to dealing with ostensibly simpler epithelial structures such as the frog skin or toad bladder these and other epithelial structures will also succumb to the following analysis and the same conclusion will be reached until new experimental approaches emerge that develop a radically different thrust

In an effort to clarify the problem equation (1) will be considered more narrowly for the circumstance where the mucosa has been short-circuited for a period of time At time zero t=0 the short-circuit I_{sc} can be abruptly reduced to 2/3 I_{sc} leading to an instantaneous voltage change E_0 As the PD follows its transient a value may be recorded at 100 milliseconds $E_{0.1}$ When the asymptote is reached we have a third value E_∞ The current is to be reduced again to 1/3 I_{sc} and finally interrupted completely so that the mucosa regains its spontaneous potential E_{sp} At each step when the current is reduced by 1/3 of the original I_{sc} the PD is allowed to traverse a full transient until a new steady state is reached At each step we record a new voltage developed instantaneously" E_0 the value at 100 milliseconds $E_{0.1}$ and finally the new steady state level E_∞ The resultant values of I and E allow us to plot three straight lines portrayed diagrammatically in Figure 7

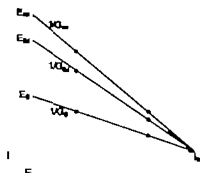


Figure 7 Epithelial conductance lines

A diagrammatic illustration of conductance lines expected of an epithelium (as reciprocals) In steps the short-circuit current is reduced by a third At each current step change the PD passes through a transient and is recorded as E_0 when t=0 $E_{0.1}$ t=0 1 sec and as E_∞ for the new steady state (E_0 of the next step) On completion the spontaneous PD is recorded as E_{sp} Reciprocal slopes for conductance are obtained for $1/G_0$ when $t=0$ $1/G_{0.1}$ t=0 1 sec $1/G_\infty$ $t=\infty$

Such conductance lines could arise from any linear array that may be catenary mammillary or extended mammillary (18) A simple series or catenary array is depicted in Figure 8

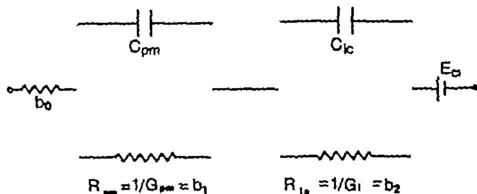


Figure 8 The transient equivalent circuit for a series dry-cell battery

Similar to previous subcircuits The subscripts for capacitors C and resistors R are pm for a dielectric plasma membrane and *lc* for an ionic-capacitor' A chloride "pump' is given as E_{CL} and b_0 is a series resistance

For this example assign values to the several components of the plasma membrane or dielectric and ionic subcircuits so that the half times become respectively 1 millisecond and 10 seconds At 1 millisecond (t=0 or "instantaneous") the resistance of the dielectric subcircuit is 0 1%R_{pm} that of the ionic subcircuit infinitesmal and for reasonable purposes $R_0 = b_0$ At 100 milliseconds the resistance of the dielectric subcircuit is 99%R_{pm} and that of ionic-capacitor 0 1% of R_{lc} so that for practical purposes $R_{0\ 1} = b\quad b_1$

We now have for the series array the conductances

$$\text{"instantaneous"} \qquad G_0 \quad 1/(b_0) \tag{8}$$

$$\text{"100 milliseconds"} \qquad G_{0\ 1} = 1/(b_0 + b_1) \tag{9}$$

$$\text{"stead state"} \qquad G_\infty \quad 1/(b_0 + b_1 + b_2) \tag{10}$$

However in the mammillary array by virtue of the *first interaction* there may be many and perhaps very many many exponential terms whose apparent half times are 1 millisecond or less Thus in the extended mammillary universe

$$G_0 = 1/(b_0 + \Sigma\, b_i') \tag{11}$$

$$= a_0 \tag{12}$$

where Σb_i is the sum of the many phantom intercepts While values for individual intercepts b_i would become vanishingly small as the aggregate number of displaced terms becomes large there is no rational basis for concluding that

$$b_\theta >> \Sigma b_i'' \tag{13}$$

Until this issue is disposed of we must conclude that a_θ of equation (1) is not synonymous with a pure non reactive series resistance and the instantaneous' conductance is without meaning

As we extend the mamillary system the *second interaction* allows of the possibility that

$$G_{\theta\ 1} = 1/(a_\theta + b_1 \quad b_2 + b_3 \quad) \tag{14}$$

with a_1 b_1 etc but with it not being known whether any particular b_i arises from an ionic-capacitor or a dielectric subcircuit Thus if $G_{\theta\ 1}$ is intended to include all values of R_{pm} but exclude those of R_{io} then $G_{\theta\ 1}$ is without merit

There remains the disparaged steady state-conductance G_∞ As a single parameter it may be inadequate for the Herculean task of unlocking the black box but it is a valuable parameter It is the only parameter applicable to isotopically measured partial conductances It is the linear parameter that gives the net ionic movement as a function of the transmural potential difference It may not be the most attractive epithelial conductance but it is the only one we have

While it is the transmural ionic conductance it would be premature to speak of it as the sum of all transmural partial ionic conductances implying that each partial conductance is linear when we lack such evidence

SUMMARY

I To facilitate commentary on electrical transients displayed by epithelial structures and to define the conductance of the latter two new terms have been introduced

a) the *pseudo-exponential* transient for the linear symmetrical transient of many epithelial structures

b) an *ionic-capacitor* for units which have the capacitance properties of a polarizable electrolyte compartment

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ADDENDA

CAVEAT #1 I am indebted to my colleague Professor R.C Arzbaecher who on short notice developed Laplace transforms for the time-constants of the transients obtained by current-clamping and by voltage-clamping In the case of the equivalent circuit given in Figure 6 (as well as that of Figure 3 Rehm W S and Tarvin J T this Symposium) the naked simplicity reveals that voltage-clamping is equivalent to radical surgery We no longer have a mammillary array the RC sub-circuits are uncoupled and the half times become $t_{\frac{1}{2}} = 0\ 7R_1C_2$ and $t_{\frac{1}{2}}$ $0\ 7R_2C_2$ In real life there has to be provision for a series resistance R_s e g between the two resistors $\frac{1}{2}R_3$ and the lower accessible-terminal As we are presently able to study epithelia we are actually evaluating physiological mucosae (or the equivalent thereof for the frog skin and others) as emphasized in the legends for Figure 3 To what extent the transients obtained by voltage-clamping and current-clamping are differentiated Figure 6 or are superimposable depends on whether R_s is negligible or largely relative to the other resistances

CAVEAT #2 While the issue of the meaning of conductance is clearly paramount for the gastric mucosa and other epithelia it is sensible that semantic clarity is required in the study of single cells such as the erythrocyte black lipid films or beyond biology in the use of the Wheastone bridge

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Effect of Posterior Lobe Hormones on the Isolated Frog Gastric Mucosa

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ABSTRACT The antidiuretic hormone (ADH) was added to the isolated gastric mucosa of frogs mounted as a membrane in a two-chamber system A concentration of ADH as small as 3 5 10 ^{7} M showed a profound inhibition of the hydrogen ion secretion in a spontaneously secreting mucosa and sometimes in a pentagastrin stimulated one but was without effect if the secretion was induced ty histamine cyclic AMP or aminophylline Oxytocin showed identical effect The effect does not seem to depend on an inhibition of histamine liberation The transmucosal DC-resistance showed a slight increase

The earliest report on inhibitory action of posterior lobe extracts on gastric secretion appeared in 1936 (1) but was soon followed by others (2 8) Most of these authors used purified or synthetic ADH preparations One paper (4) also described the inhibitory effect of oxytocin

Karlmark & Öbrink (7) claimed that effect was obtained with dosages of ADH which were considered to give concentrations in blood and tissue within physiological limits indicating that ADH may play a physiological role in the control of the gastric secretion Similar conclusions were drawn by Abelson & Zaks (9)

Therefore it seems possible or even probable that ADH exerts its inhibitory actions not by its vasoactive principle but by some other means (metabolic or membrane interference)

No reports have appeared on the effect of ADH or oxytocin on the gastric secretion in isolated stomachs or gastric mucosa It was therefore considered of fundamental interest to study the effect of pure posterior lobe preparations on the isolated gastric mucosa of frog that has been the most widely used preparation

Methods

General procedure Frogs (Rana Temporaria) were kept in a tank containing running tap water of 16°C During the first part of the series of experiments the animals were force fed with pieces of liver once or twice a week Later on the frogs were allowed live food (newborn mice)

The frog was decapitated the stomach cut out and the mucosa isolated and cut open The mucosa was mounted between two Perspex chambers containing 10 ml of solution each with an exposed area of 1 8 cm^2 The nutrient solution contained 81 6 mM NaCl 3 2 mM KCl 1 8 mM $CaCl_2$ 0 8 mM $MgSO_4$ 0 8 mM KH_2PO_4 17 8 mM $NaHCO_3$ 3 0 mM $NaCH_3COOH$ and 2 0 mM Glucose The solution on the secretory side contained 102 4 mM NaCl 4 0 mM KCl 1 8 mM $CaCl_2$ and 0 8 mM $MgSO_4$ Both chambers were gassed with 5% CO_2 and 95% O_2 which performed the stirring of the chambers The experiments were performed at room temperature

Some experiments were performed in a dual two-chamber system where one mucosa was mounted such as to constitute the partitions in two parallel systems One side could then be used as a control for the other one

On the secretory side the pH was kept constant at 4 7 by a pH-stat method with a solution containing 84 8 mM NaCl 1 3 mM Na_2SO_4 4 0 mM KCl 15 0 mM NaOH and 7 9 mM Mannitol for titration

Electrical parameters The transmucosal PD was measured between two calomel electrodes supplied with 1 M KCl agar bridges The experiments were performed without short-circuiting the mucosae

The resistance across the mucosal membrane was determined by sending a squarewave of 100 μA current through the mucosa first in the direction mucosa-serosa for 30 msec and then in the opposite direction for another 30 msec (10) During this time the PD changed to a new level which appeared to be a steady one The Δ PD was used for calculating the resistance

The current generated by the mucosa (estimated short-circuit current) was calculated as (PD) divided by (Resistance)

Drugs To stimulate the secretion the following substances were sometimes added to the nutrient side

a) histamine dihydrochloride to a final concentration of 10^{-4} M

b) pentagastrin (MW=772) (Peptavlon^R ICI Pharma) to a final concentra

tion of 6 x 10^{-4} M

c) Aminophylline (MW=420 4) (Sigma) to a final concentration of 6x10^{-4}M

d) N^6 O^2-dibutyryl-c-AMP (MW=491 3) (Sigma) to a final concentration of 10^{-3} M

Vasopressin was obtained from Sandoz Ltd as synthetic lysinvasopressin The concentration of vasopressin is normally given as IU defined as antidiuretic or vasopressive effects In the present study it is more appropriate to express the concentration in moles/l (MW=1056) For the preparation at hand one IU was equal to 3 5x10^{-9} moles of vasopressin which was added to the nutrient (or in some cases the secretory) side giving a final concentration of 3 5x10 ^{7}M Oxytocin (MW=979) was obtained from Ferring Ltd and used in a similar fashion as vasopressin The normal dose added contained 1 IU corresponding to 2 6x10 ^{9} moles giving a final concentration of 2 6x10 ^{7} M

Histamine determinations. Histamine was determined in the nutrient and secretory solutions with the fluorescence method of Shore et al (11) modified by Håkanson & Rönnberg (12) using a ratio fluorometer (Farrand Optical Co) 2 ml of the sample were mixed with 0 4 ml 1 M NaOH and 0 1ml 0 4% O phtalaldehyde The condensation step was carried out in N_2-atmosphere at -20°C overnight 0 25 ml of 0 8 or 0 9MH_2SO_4 were then added during thawing and the degree of fluorescence read within a few hours

Results

The mucosae did not behave uniformly Some showed a spontaneous secretion of HCl whereas others were in or near secretory rest All the mucosae could be stimulated by some secretagogue added to the nutrient side

When the mucosa showed a steady secretion rate vasopressin was added to the nutrient side The effect of the ADH varied. In experiments with spontaneously secreting stomachs a marked inhibition of the acid secretion appeared within a few minutes whereas in histamine stimulated stomachs the inhibition was small or entirely absent This is seen in fig.1 where many experiments are put together

The average figure for the H^+-secretion rates was 2 41 µeq cm ^{2} hr ^{1} for the spontaneously secreting stomachs and 3 00 ueq cm^{-2} hr^{-1} for the histamine stimulated ones After ADH-administration (time = zero)

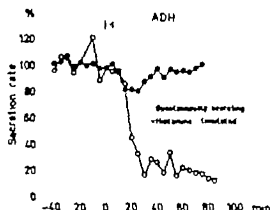


Fig 1 A summary of several experiments The pre-ADH secretion rates were normalized to 100% ADH was in all experiments added to a final concentration of 3 5 x 10^-7 M

the average secretion rates from 25 minutes and thereafter were 21% of the original rate for the spontaneously secreting stomachs and 91% for the histamine stimulated ones

It was made sure that the solvent for ADH (a mixture of alcohol Na-acetat acetic acid and chlorbutol) did not interfere with the results

There may be an important pitfall in comparing inhibitory effects if the preinhibitory stimulation is not well defined Öbrink (13) showed that for the same dose of inhibitor the inhibition could be almost 100% when the secretion rate in the stomach before the inhibition was low but could decrease to almost nil when the preinhibitory secretion rate was at its highest level It was therefore necessary to compare the secretion rates in the two groups before the administration of ADH In fig. 2 the preinhibitory secretion rates are presented and it is evident that even if in some experiments the initial secretion rates were lower in the spon

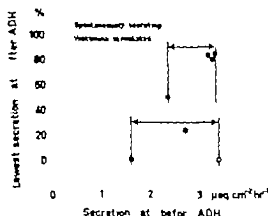


Fig 2 The histamine induced secretion was very much less inhibited by ADH than the spontaneous one This was not however due to different pre-inhibitory secretion rates as can be seen from this figure

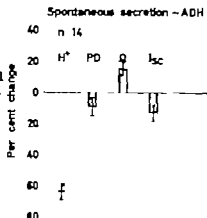


Fig 3 The percentage change of H^+ secretion rate transmucosal potential difference DC-resistance and calculated short circuit current after ADH administration to spontaneous secretion The vertical lines denote ± S E of the mean

taneously secreting stomachs than in the histamine stimulated ones the two groups covered the same order of secretion rates Fig 2 also shows the degree of inhibition as represented by the lowest post ADH value obtained There was no tendency to a stronger inhibition of lower preinhibitory secretion rates Thus the difference in inhibition between the two groups cannot be accounted for by different preceding stimulation rates The percentage inhibition for a whole group of experiments with spontaneous secretion is shown in fig. 3 and after histamine stimulation in fig. 4

Histamine is thought to act through the activation of adenylate cyclase. Consequently the effect of ADH on the cyclic AMP-stimulated secretion was also investigated A clear but not excessive stimulation of acid output was obtained with dibutyryl-c-AMP at a final concentration of 10^{-3}M After this treatment ADH was as ineffective as inhibitor as after histamine Similar results appeared when the secretion was induced by the phosphodiesterase inhibitor aminophylline at a final concentration of $6x10^{-4}$M – fig 4

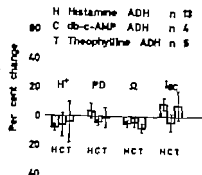


Fig 4 The percentage change of H^+ secretion rate transmucosal potential difference DC-resistance and calculated short circuit current after ADH administration to secretion induced by histamine c-AMP or aminophyllin The vertical lines denote ± S E of the mean

When gastrin was used for stimulation however the results sometimes resembled the ones obtained with ADH on spontaneous secretion fig. 5 These results were however not consistent The inhibitory power never appeared if other types of treatment had been given to the mucosa prior to the gastrin-ADH assay

Effect on electrical parameters.
In order to further elucidate the mode of action of ADH on the gastric mucosa the transmucosal PD and the electrical resistance were recorded The calculated short circuit current was obtained as the quotient between the PD and the resistance The electrical parameters (average values) are presented in Table I The percentage change was calculated for each experiment separately Therefore the figures for change % in the table may not agree with the quotient of the averaged figures after ADH / before ADH The changes in per cent of pre-ADH-values are visualized in figs 3 4 and 5 No drastic changes occurred as a result of ADH-administration with the possible exception of the resistance in the case of spontaneous secretion where the resistance increased As the PD was unchanged the generated current decreased Fig. 6 shows this in an individual experiment

Oxytocin as an inhibitor
Experiments with oxytocin were performed in similar ways as with ADH Fig. 7 summarizes the effects of oxytocin on spontaneous as well as histamine stimulated secretion The effect of oxytocin resembled that of ADH but the changes of the electrical parameters were even less pronounced No decrease in I_{sc} was detected but possibly an increase in PD

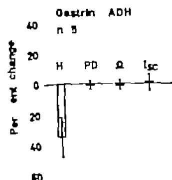


Fig 5 The percentage change of H^+ secretion rate transmucosal potential difference DC-resistance and calculated short circuit current after ADH administration to secretion induced by pentagastrin The vertical lines denote ± S E of the mean

Table 1 The steady state secretion rates and electrical parameters and their changes after ADH administration in spontaneously secreting or stimulated gastric mucosae

Stimulation	Number of experiments	Steady state secretion rate of H^+ Average $\mu eq\ cm^{-2}\ hr^{-1}$ before ADH	after ADH	change %	Steady state PD Average mV before ADH	after ADH	change %	Steady stat resistance Average $\Omega\ cm^2$ before ADH	after ADH	change %	Steady state generated current Average $\mu eq\ cm^{-2}\ hr^{-1}$ before ADH	after ADH	change %
Spontaneous	14	1 67	0 63	-64	16 62	15 27	8	323.64	364.53	+15	1 82	1 68	13
±SD		0 58	0 44	18 97	4 06	5 18	22,62	86.73	76.92	17 07	0 80	0 71	20 44
±SE		0 15	0 12	5 24	1 08	1 38	6 10	22.9	20.6	4 56	0 21	0 19	5 47
Histamine	13	2 55	2 32	-8	13 01	13 46	+4	321.48	321.54	5	1 58	1 77	+9
±SD		0 54	0 47	8 12	3 90	4 58	15,55	98.56	104.28	9 57	0 69	0 85	16 37
±SE		0 15	0 13	2 25	1 08	1 27	4,31	27.34	28.92	2 37	0 19	0 24	4 53
db-c-AMP	4	1 28	1 24	-6	15 96	15 4	-4	297 0	284.5	-4	2 23	2 11	-4
±SD		0 29	0 50	15 63	3 63	4 01	4 43	50.58	46.68	5 91	0 77	0 72	11 85
±SE		0 15	0 25	7 82	1 62	1 79	2 22	25.29	23.33	2 98	0 39	0 36	5 93
Theofyllamin	5	1 68	1 59	-4	13 0	12 80	1	359.60	329.80	-8	1 42	1 50	+6
±SD		0 40	0 54	32 26	3 05	3 17	13 44	40.19	50.16	6 61	0 52	0 47	21 39
±SE		0 18	0 24	14 40	1 36	1 42	6 09	29.92	22.43	2 93	0 23	0 21	9 55
Pentagastrin	5	2 31	1 82	35	16 12	16 04	1	319.0	315.0	1	1 90	1 90	+1
±SD		0 65	0 96	28 82	2 92	3 34	6 10	32.53	35.92	8 35	0 57	0 53	11 97
±SE		0 29	0 43	12 87	1 31	1 49	2 72	14.54	16.06	3 73	0 25	0 24	5 34

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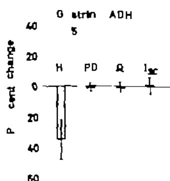


Fig 5 The percentage change of H^+ secretion rate transmucosal potential difference DC-resistance and calculated short circuit current after ADH administration to secretion induced by pentagastrin The vertical lines denote ± S E of the mean

kinase which is not activated by histamine (14) In the toad bladder ADH causes aggregation of intramembranous particles in the luminal membranes of granular cells thereby influencing the osmotic water flow (15) The inability of ADH to inhibit gastric secretion stimulated by exogenous histamine or c AMP (or aminophyllin) may indicate that these substances prevented the specific ADH function in the cell It may also suggest that the stimulating mechanisms for the spontaneous secretion are different from those induced by exogenous histamine This is of course highly hypothetical but cannot be ruled out at the present time

Preliminary experiments with ADH on isolated gastric glands (16) indicate that the inhibition is not accompanied by a decrease in oxygen consumption

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Gastric H^+ Ion Secretion in the Isolated Whole Rat Stomach–The Effects of Agonists and Antagonists

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Studies on gastric H^+ ion secretion in vivo are complicated by hormonal neural and vascular influence The use of isolated parietal cell preparations removes these influences but H^+-ion transport cannot be measured directly oxygen consumption or aminopyrin accumulation being used as indirect measurements Studies on n isolated whole stomach preparation provides a bridge between sults obtained in vivo and those obtained on isolated pari tal cell preparations The present paper d scribes studies arried out on an isolated whol stomach preparation from an immature rat the lumen of the stomach being perfused and the H^+-ion activity of the perfusate ecorded continuously

The preparation has basal secr tion (mean from 21 pr parations of 4 86 mol x 10^{-8}/min) which remains stabl over a 6 hour period The origin of thi secr tion i not clear but it is not inhibited by the histamine H_2-receptor antagonist metiamide at concentration as high 10 ^{3}M It i however educed by high concentrations (10^{-4}M) f th anticholinergic drug tropin and by thiocyanate at 10 mM

The pr paration gives dose-dependent H^+ ion secretory response to histamine acetylcholin gastrin and dibutyryl cyclic adenosine 3 5 monophosphate (db cAMP) and the responses are readily reversibl on washing out of the agonist However with all th agonists the secretory response showed fade when th agonist was allowed to remain in contact with th tissue or even when it was infused continuously into the serosal bathing fluid The fade wa least marked with db cAMP but the eason f the fad is not lear In addition th peak response to pentaga trin showed marked tachyphylaxi on repeated administration The othe agonists did not exhibit thi tachyphylaxis

Antagonist studies with metiamide showed that in the concentration rang 3×10^{-6}M - 3×10^{-5}M th compound inhibited histamine-stimulated secretion The antagonism appeared to be of a competitiv type and the results suggested a homogeniety between the histamin H_2 receptors on the g stric mucosa and those on other tissu s such as the guinea-pig trium and rat uteru

Th secretory response to gastrin was inhibited by the same concentration rang of metiamid b t the maximum response was depressed a phenomenon also observed in vivo in the r t dog and man

Concentration of metiamide as high s 10^{-3}M failed to inhibit gastric H^+-ion secretion stimulated by acetylcholine sugge ting that histamine is not involved in the esponse to thi secretagogue Also high concentrations of metiamide (upto 10^{-3}M) failed to inhibit db cAMP-stimul ted secretion The results with db cAMP suggested that it acted at a point distal to the histamin H_2 ecepto

Atropin at 10^{-3}M failed to inhibit histamine-stimulated secretion Significant inhibition of acetylcholine-stimulated secretion by atropin wa obtained in the concentration ange 10^{-6}M 3×10^{-6}M but these concentrations are high compared to those used to block muscarinic recepto fo exampl on th guinea-pig ileum (10^{-9}M 10^{-8}M) Atropin at 10^{-5}M failed to inhibit gastrin-stimulated secretion but significant inhibition was found at 10^{-4}M and 10^{-3}M

Overall th results could be interpreted a indi ating that the rat gastric mucosa contain histamine H_2 receptors and acetylcholine receptors but that gastrin cts at least in part via a histaminergic pathway Th failure of very high concentrations of metiamide to inhibit acetylcholine-stimul ted secr tion and of high concentrations of tropine to inhibit histamine-stimulated secr tion argues again t the hypothe is of receptor interaction put forward by Grossman and Konturek at least in the at gastri mucosa in vitro

Pr liminary studies on th ionic dependance of gastric H^+-ion secretion hav hown that removal of Ca^{++} ions from both the mucosal and serosal bathing fluids enhance the than reduce th response to histamine In addition basal secr tion was not reduced a esult which contra ts with th sults of other workers using amphibian mucosa in vitro Clearly studies using Ca^{++} chelating agents such as EGTA are equired

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Interpretation of the Voltage Response of Epithelial Tissue to Step Currents

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Abstract: The transient voltage response of the gastric mucosa under constant current conditions is examined in terms of a mathematical model It is shown that the surface cells cannot be ignored in such an analysis in spite of the fact that their resistance may be five-fold or more that of the tubular cells In particular there is a time constant present in the analyzed response which may be an order of magnitude less than the time constant of any of the cell membranes

Electrical resistance measurements have been very useful in elucidating the mechanisms of ion transport in many tissues For example it has been shown (1 2) for the frog gastric mucosa by means of resistance measurements that most of the Cl^- and all of the HCO_3^- move across the nutrient membane (the membrane facing the submucosa) by means of a neutral (nonconductive) mechanism (or mechanisms)

Certain problems arise in the determination of the electrical resistance of biological systems and in this paper some of these problems will be analyzed Although our interest is primarily concerned with the frog s cornea and the frog s gastric mucosa we believe that our analysis is of general applicability to epithelial tissues

For an initial approach to the problem an idealized system is examined in which there is a flat sheet of cells with the resistance between the cells (the transintercellular region) having an essentially infinite resistance i e all of an applied current would traverse the plasma

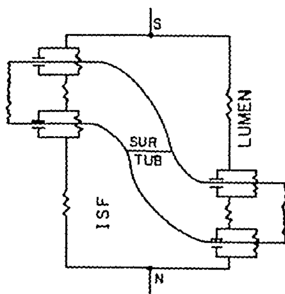


Fig 2 Equivalent circuit for the frog gastric mucosa The left-hand limb represents the lumen and nutrient membranes of the surface cells (SUR) while the right-hand limb represents the membranes of the tubular cells (TUB)

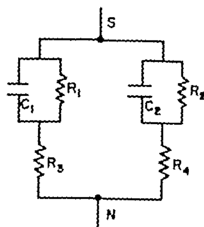


Fig 3 Simplified equivalent circuit for the frog gastric mucosa By assuming the same time constant for both the lumen and the nutrient membranes in Fig 2 it becomes possible to represent both the surface and the tubular cell membranes by the subcircuits (R_1 C_1) and (R_2 C_2) respectively See text

with the T s and hence with values of the R s and C s other information would be needed For example if it were known that a given agent or procedure would change only the characteristics of one of the membranes so that its time constant were changed significantly then a transient obtained under the new experimental conditions would result in a change in one of the time constants and no change in the other time constant Hence the R s and C s of the two membranes would then be known

If T_1 and T_2 were the same under standard conditions but under new experimental conditions were different then under the new experimental conditions (if it were again known that only one of the membranes was changed) the R s and C s of each membrane could be easily determined

The above analysis is for the simplest of epithelial systems Interpretations of the voltage transient of less simple systems is far more difficult Still the goal in studies of the voltage transients to step currents in complex tissues is (a) the determinations of the R s and C s of each membrane of the tissue and (b) the mechanisms by which each ion is transported across the membranes whether it be via conductive channels or nonconductive mechanisms or by passive or active mechanisms Our primary goal in this paper is the presentation of certain problems that arise in the analysis of transients in complex tissues

IN VITRO FROG GASTRIC MUCOSA

Figures 2 and 3 represent lumped parameter circuits for the in vitro frog gastric mucosa The left-hand limb of Fig 2 represents the surface cells and the other limb the tubular cells We should point out that due to the complex geometry of the mucosa a distributed parameter circuit would undoubtedly more adequately represent this tissue However on the basis of present knowledge it would be ridiculous to attempt to use a distributed parameter circuit Therefore for the present we are constrained to use a lumped parameter circuit for the analysis of the voltage transients It should be noted that there are

essentially two cell types in the frog gastric mucosa i e the surface cells which line the surface and the pits and the tubular cells which line the tubules Since each cell type has a lumen membrane and a nutrient membrane there are 4 plasma membranes It is important to point out that even for a complex tissue the series resistance R_S and the total resistance can still be easily obtained

There is evidence indicating that the resistance through the surface cells is substantially greater than the resistance via the tubular cells and the lumina (7 8) If we assume that the resistance through the surface epithelial cells is five or more times that through the tubular cells plus lumina then the total resistance would give to a good first approximation the resistance through the tubular cells and lumina If we studied changes in the total resistance and as long as the resistance via the surface cells was still substantially greater than that via the tubular cells then changes in the total resistance could be interpreted to a good first approximation as due to changes via the tubular cells In other words for certain analyses we could disregard the surface cells However in the analysis of the transient voltage responses we will show that even with the surface cell resistance substantially greater than that of the tubular cells we cannot ignore the resistance of the surface cells We will show in the analysis of the voltage transients that at least one of the derived time constants (i e the time constants for the total circuits of Fig 2 and 3) may be much less than any of the time constants of the individual membranes We should point out that for the in vitro frog gastric mucosa the voltage response (in the msec range) cannot be represented by an equation with a single time constant but can be quite accurately represented by an equation with two time constants (6) In contrast for an accurate representation of the voltage transient of the frog s cornea more than two time constants are required (9) For the analysis of the voltage transient of the frog

gastric mucosa we used initially a simple stripping technique (4) and later a more accurate technique (6) With both techniques we found two time constants which were about an order of magnitude apart For the longer time constant with the stripping technique a value of the capacitance of about 400 µf cm^{-2} of macroscopic area was obtained but with the more sophisticated and more accurate technique the value of the capacitance for the longer time constant was substantially less being in the range of about 100 µf cm^{-2} The actual surface area of the nutrient membranes of the surface and tubular cells and the secretory membrane of the tubular cells is in the neighborhood of about 100 times the gross macroscopic area (10)

Returning now to our main theme we have obtained a closed form solution of the circuit of Fig 2 but a presentation of this aspect of our analysis is beyond the scope of the present paper Obtaining a closed-form solution involves the solution of a fourth order linear differential equation and the manipulations are quite tedious In order to illustrate the thrust of our analysis we will make the simplifying assumption that the two time constants in the left limb of the circuit in Fig 2 are the same and that the two time constants in the right limb are the same On the basis of this assumption the circuits in Fig 2 can be reduced to the circuit shown in Fig 3 Now the solution of this circuit involved only the solution of a second order linear differential equation (which can be easily obtained from our solution of the fourth order differential equation of the circuit in Fig 2)

Solution of the second order differential equation shows that the voltage response V_{NS} to a step current of constant magnitude for the circuit in Fig 3 is given by:

$$V_{NS} = IR_0 + IR_1(1-e^{-t/T_1}) + IR_2(1-e^{-t/T_2}) \qquad (3)$$

where

$$R_0 + R_1 + R_2 = R_\infty \tag{4}$$

and R_0 is given by:

$$R_0 = \frac{R_3R_4}{R_3+R_4} \tag{5}$$

R_∞ defined by equation (4) is given by V_{NS}/I when $t \to \infty$:

$$R_\infty = \frac{(R_1+R_3)(R_2+R_4)}{R_T} \tag{6}$$

where

$$R_T = R_1 + R_2 + R_3 + R_4 \tag{7}$$

The time constants T_1 and T_2 are given by:

$$T_1 = \frac{2\alpha}{\beta - \gamma} \tag{8}$$

$$T_2 = \frac{2\alpha}{\beta + \gamma} \tag{9}$$

where α β and γ are given by:

$$\alpha = T_1T_2(R_T-R_1-R_2) \tag{10}$$

$$\beta = T_1(R_T-R_1) + T_2(R_T-R_2) \tag{11}$$

$$\gamma = \sqrt{[T_1(R_T-R_1) - T_2(R_T-R_2)]^2 + 4T_1T_2R_1R_2} \tag{12}$$

and R_1 and R_2 by:

$$R_1 = \frac{[(R_1+R_3)T_1 - R_3T_1][(R_2+R_4)T_1 - R_4T_2]T_2}{T_1T_2(R_3+R_4)(T_2-T_1)} \tag{13}$$

$$R_2 = \frac{[(R_1+R_3)T_2 - R_3T_1][(R_2+R_4)T_2 - R_4T_2]T_1}{T_1T_2(R_3+R_4)(T_1-T_2)} \tag{14}$$

Parenthetically as a first approach to the problem the reader may appreciate our use of the simplifying assumptions enabling us to use the equations for the circuit in Fig 3 rather than those for the circuit in Fig 2

It should be clear from the above that T_1 and T_2 are the time constants for the circuit of Fig 3 treated as a two terminal circuit and not the time constants for the subcircuits The time constants for the subcircuits are C_1R_1 and C_2R_2 It can be seen from the above equations that both T_1 and T_2 are rather complicated functions of all the R s and C s Equation (3) can be rearranged by use of equation (4) so that:

$$V_{MS} = IR_{\infty} - IR_1e^{-t/T_1} - IR_2e^{-t/T_2} \tag{15}$$

For the discharge after the leveling off of V during charging we have:

$$V_D = IR_1e^{-t/T_1} + IR_2e^{-t/T_2} \tag{16}$$

V_D gives the voltage after the initial step decrease ($= R_0I$) following the break of the circuit

We will now show that the voltage response of the circuit in Fig 3 may yield a time constant that is much less than that of any of the time constants of the subcircuits (i e if these subcircuits were examined in isolation by the use of step currents) To illustrate this point we let the time constants be equal for the two RC subcircuits in Fig 3 i e we let $R_1C_1 = R_2C_2 = RC$ Equation (3) after algebraic manipulations reduces to:

$$V_{HS} = R_0I + \frac{R_1R_2}{R_1+R_2}\, I(1-e^{-t/T_1}) + \frac{(R_2R_3-R_1R_4)^2}{(R_1+R_2)(R_3+R_4)R_T}\, I(1-e^{-t/T_2}) \tag{17}$$

and T_1 and T_2 are found to be:

$$T_1 = RC \tag{18}$$

$$T_2 = \frac{R_3+R_4}{R_T}RC \tag{19}$$

This result is not intuitively obvious (at least to us) but derives from straightforward algebra In other words T_1 is equal to the time constants of the two subcircuits

(when they are equal) and the second time constant is less than T_1 since obviously $(R_3 + R_4) < R_T$ Now if $R_1/R_3 = R_2/R_4$ then the coefficient of the T_2 term vanishes and the circuit would have only one time constant This latter conclusion is intuitively obvious since if $R_1/R_3 = R_2/R_4$ the circuit would be represented by a one limb circuit i e by two resistors in series with one shunted by a capacitor However if $R_1/R_3 \neq R_2/R_4$ then the coefficient will not vanish and the circuit will have two time constants

Let us examine a set of conditions where $(R_1 + R_3)$ is substantially greater than $(R_2 + R_4)$ — a set of conditions which is applicable to the gastric mucosa Assume that the R_1R_3 limb represents the surface cells and the R_2R_4 limb the tubular cells There is substantial evidence supporting the conclusion that the resistance via the surface cells is substantially greater (perhaps five-fold or more) than the resistance via the tubular cells plus lumina (7) In other words the high resistance limb represents the surface cells and the low resistance limb the tubular cells

For a specific illustration let $R_2 = R_3 = R_4 = R$ and let $R_1 = 10R_2$ (i e $R_1 = 10R$) Then T_2 from equations (18) and (19) is 0 15 T_1 Obviously it is important to determine whether the T_2 coefficient will be vanishingly small Substitution into equations (5) (13) and (14) reveals that R_0 = 0 5 R R_1 = 0 91 R and R_2 = 0 28 R In other words R_2 is about 30% of R_1 and in the analysis of the transient there would be no problem in showing that there is a time constant present which is much less than the time constant of the subcircuits

Conclusion

It should be clear to the reader that if one were not aware of the analysis presented in the foregoing one might feel constrained to attempt to identify the short time constant (including its R and C values) with a subcircuit i e with one of the membranes of the tissue For the shorter time constant such an identification would be

in error The reader may then ask about the longer time constant and the possibility of identifying it with one of the membranes An analysis of this aspect of the interpretation of the voltage transients is beyond the scope of this paper Our overall problem is a rather difficult one but with certain additional information it is within the realm of possibility that the analysis of voltage transients to step currents will result in considerable insight into the characteristics of the plasma membranes of complex tissues

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Effect of Weak Bases on Secreting and Inhibited in Vitro Frog Gastric Muocsa

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ABSTRACT: The marked decrease in measured H^+ rate which occurs when aminopyrine (AP) or imidazole (IZ) are added to the nutrient side of in vitro frog (Rana pipiens) gastric mucosa is due to neutralization of the secreted H^+ by the weak bases diffusing across the mucosa into the lumen Both AP and IZ substantially reverse thiocyanate inhibition of H^+ secretion but only partially reverse thiocyanate effects on potential difference and resistance Nitrite inhibition of H^+ secretion is not reversed by AP but occasionally is slightly reversed by IZ

This paper is concerned with the in vitro frog gastric mucosa two weak bases imidazole and aminopyrine and two inhibitors of H^+ secretion thiocyanate and nitrite Alonso et al reported that imidazole and certain of its congeners markedly reduced the measured H^+ rate of the in vitro frog gastric mucosa (1) We confirmed this finding for imidazole and found that aminopyrine would also reduce the measured H^+ rate Both of these weak bases produced changes in the electrophysiological characteristics of the mucosa We report here that the reduction in measured H^+ secretion is caused by the weak bases diffusing in neutral form across the cell layer and neutralizing the secreted H^+ ions in the lumen

In the course of these experiments thiocyanate was used to test the validity of H^+ rates and we report here that both aminopyrine and imidazole will reverse thiocyanate inhibition of H^+ secretion

In further experiments we found that aminopyrine would not reverse nitrite inhibition of H^+ secretion and that imidazole would occasionally reverse it slightly Preliminary reports of these results have been presented elsewhere (2 3)

Methods

The experiments were performed on Rana pipiens with an in vitro method described in detail elsewhere (4) The external muscle layers were removed from the frog stomach and discarded The resulting preparation referred to as the gastric mucosa consists of the mucous coat the invaginated continuous cell layer (one cell thick) the lamina propria the muscularis mucosa and part of the submucosa The gastric mucosa was mounted between chambers and the H^+ secretory rate measured by means of the recording pH stat method in which the pH of the fluid on the secretory (mucosal) side was maintained at an appropriate value Two pairs of electrodes were used one pair for sending current across the mucosa and the other pair for potential difference (PD) measurements The PD was measured with a recording potentiometer with an 0 125 sec full-scale response The resistance was determined as the change in PD (0 5 sec after the application of current (5)) divided by the current Resistances are given as resistance between the two probe electrodes

The fluids on the secretory and nutrient (submucosal) sides were connected to their own reservoirs and circulation was by gas lifts The secretory side was gassed with 100% O_2 and the nutrient side with 95% O_2 – 5% CO_2 Composition of the Cl^- secretory fluid was (mM): Na^+ 100; K^+ 4; Cl^- 104 The composition of the Cl^- nutrient fluid was (mM): Na^+ 102; K^+ 4; Ca^{++}1; Mg^{++} 0 8; Cl^- 81; $SO_4^=$ 0 8; HCO_3^- 25; phosphate 1 0; glucose 10; histamine 0 1 The weak bases imidazole or aminopyrine were always added to the nutrient solution Thiocyanate (NaSCN) was added to the secretory side except for one series of experiments noted below Statistical analyses were by the paired t-test

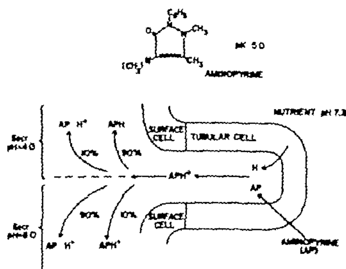


Fig 1 Schematic diagram of gastric tubule illustrating neutralization hypothesis

Preliminary studies

To determine whether the weak bases inhibited or neutralized H^+ secretion we used the method illustrated in Fig 1 for aminopyrine (pK_a = 5) The neutral form of aminopyrine (AP) diffusing across the cell into the lumen would pick up a secreted H^+ ion and enter the secretory bathing solution in the charged form (APH^+) If the secretory pH = 4 then 90% would remain in the charged form (APH^+) and 10% would give up a H^+ to be titrated and the measured H^+ rate would decrease markedly If however the pH of the secretory solution was 6 0 only 10% would remain in the charged form (APH^+) and 90% would release its H^+ and the measured rate would hardly be affected For imidazole (IZ) (pK_a = 6 95) with the secretory pH = 4 only 0 1% of the charged form (IZH^+) would release a H^+ ion and changing the secretory pH to 8 should restore the H^+ rate to 90% of control

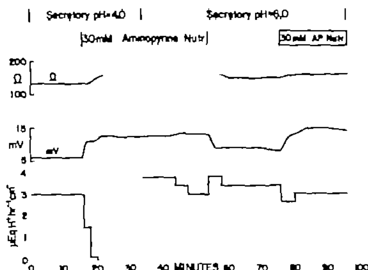


Fig 2 Effect on H^+ PD (mV) and resistance (Ω) of changing from secretory solution of pH 4 to one of pH 6 in presence of 30 mM aminopyrine

Results

Effect of changing secretory pH

In Fig 2 we see that when the secretory pH is maintained at 4 0 the addition of 30 mM aminopyrine to the nutrient side caused the measured H^+ rate to drop to zero with a slight rise in PD and resistance When the secretory fluid was changed to one with a pH of 6 0 there was an immediate return of the measured rate but no change in PD or resistance When the aminopyrine was removed the H^+ rate continued at about the same level while PD and resistance decreased to control values The subsequent re-addition of 30 mM aminopyrine with the pH of the secretory solution at 6 had little effect on the H^+ rate although again the PD rose In a series of 7 experiments with the secretory fluid pH = 4 0 the measured H^+ rate decreased by 98% (SD ± 4 5) and upon changing to a secretory fluid of pH 6 the H^+ rate was 115% (SD ± 30 8) of control When the aminopyrine was removed the H^+ rate was also 115% (SD ± 21 6) of the control rate

In 8 experiments where the secretory fluid was maintained at pH = 6 throughout the addition of 30 mM aminopyrine to the nutrient fluid caused a small but significant decrease in H^+ rate (P 0 02) when compared to the initial control rate However there was no difference when compared to the final control The PD in aminopyrine was significantly higher when compared to either control (P 0 001) while the resistance showed no significant change

In experiments in which 20 mM imidazole was added to the nutrient fluid similar results were found In 8 experiments with the secretory pH maintained at 4 0 the measured H^+ rate was reduced by 88% (SD $\pm$ 21 6) and when the secretory solution was changed to one with a pH of 8 the H^+ rate was 156% (SD $\pm$ 30 9) of the control When the imidazole was removed the H^+ rate was 114% (SD $\pm$ 13 0) of the control rate

In 11 experiments in which the secretory pH was fixed at 8 0 the average H^+ rate following the addition of 20 mM imidazole to the nutrient fluid increased but was not significantly greater than the initial or final control Both the PD and resistance were significantly greater than either of the controls

Reversal of thiocyanate inhibition

Thiocyanate (5-15 mM) added to either the secretory or nutrient solution inhibits H^+ secretion increases PD and resistance and these effects are reversed when thiocyanate is removed (4) These effects of thiocyanate occur regardless of whether the secretory pH is 4 0 6 0 or 8 0

In order to test the validity of the H^+ rates seen at the higher secretory pH s with the weak bases we added thiocyanate to the secretory solution when either 20 mM imidazole or 30 mM aminopyrine was on the nutrient side and saw very little decline in H^+ rate This unexpected finding led to a series of experiments which disclosed that both aminopyrine and imidazole would reverse thiocyanate inhibition of H^+ secretion—a surprising result

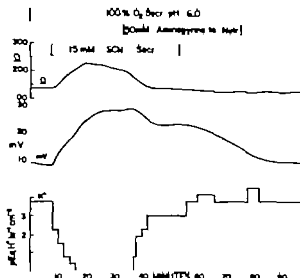


Fig 3 Effect of 30 mM aminopyrine in presence and absence of 15 mM thiocyanate on resistance (Ω); PD (mV); H^+ rate

Figure 3 illustrates this point The pH of the secretory solution was maintained at 6 0 throughout since as we have just shown H^+ rates with aminopyrine cannot be measured at low secretory pH s Thiocyanate (15 mM) was added to the secretory side and its typical effects are seen The PD rises the resistance increases and the H^+ rate goes to zero With thiocyanate still present the addition of 30 mM aminopyrine to the nutrient side restores the H^+ rate to about 70% of control The PD declines slightly but remains well above control levels In this particular experiment the resistance did return to control levels with restoration of the H^+ rate but this was not a consistent finding

Removal of the thiocyanate from the secretory side with aminopyrine still present in the nutrient produced a small increase in H^+ rate little change in resistance and a gradual decline of the PD to about the control level before the addition of thiocyanate Removal of the aminopyrine had little effect

In 7 experiments the H^+ rate was inhibited an average of 94% by thiocyanate aminopyrine restored the rate to 70% of control The average decrease in PD upon the addition of aminopyrine to a thiocyanate-inhibited stomach was about 10% the average decrease in resistance about 20%

Similar results were obtained with experiments like Fig 3 in which the secretory pH was maintained at 8 0 The H^+ rate after inhibition by thiocyanate (added to the secretory side) was restored by addition of 20 mM imidazole to the nutrient side In a series of 6 experiments the H^+ rate was reduced 87% by thiocyanate and 20 mM imidazole restored the average H^+ rate to 90% of control Although imidazole produced almost complete reversal of the H^+ inhibition it did not appreciably reverse the effect of thiocyanate on the PD and resistance In 7 experiments in which the thiocyanate was added to the nutrient side and the imidazole was subsequently also added to the nutrient side the results were essentially the same Imidazole caused almost complete restoration of the thiocyanate-inhibited H^+ rate but did not produce much change in the thiocyanate-elevated PD or resistance

The question naturally arises as to whether these restored H^+ rates are true H^+ secretion We found that the H^+ secretion seen in the presence of thiocyanate and aminopyrine (or imidazole) could be abolished by making the tissue anoxic or by adding amytal (8 mM) to the nutrient solution With either agent the restored H^+ rate was reduced to zero the PD fell and resistance rose and all parameters could be re-established following return to oxygen or removal of the amytal Thus the H^+ rate seen when the weak bases reverse thiocyanate inhibition is true H^+ secretion

If we first inhibit the H^+ rate with anoxia or amytal neither aminopyrine nor imidazole will restore the H^+ rate Therefore the weak bases are not in some way picking up a H^+ ion from the cytoplasm and taking it out into the lumen

Nitrite experiments

The mechanism by which thiocyanate inhibits H^+ secretion is still not known Some years ago Rehm and co-workers (6) reported that at similar concentrations nitrite cyanate and ammonium as well as thiocyanate suppressed gastric secretion All four of these ions possess a pair of unshared electrons on a nitrogen and they proposed that this property was responsible for the inhibition of H^+ secretion

Since we found that aminopyrine and imidazole would reverse thiocyanate inhibition of H^+ we next tested the effects of these weak bases on nitrite inhibition

Sodium nitrite (15 mM to nutrient) inhibits H^+ secretion and we found in 10 experiments that aminopyrine (up to 60 mM) would not reverse nitrite inhibition In 6 of 13 experiments with imidazole there was no reversal of nitrite inhibition while in 7 experiments there was an average of 28% return of H^+ rate Thus there are similarities but also differences between the effects of the weak bases on thiocyanate and nitrite inhibition

Experiments in chloride-free media

Experiments were also performed with Cl^--free media and the results are of considerable interest with respect to the effects of these weak bases on thiocyanate inhibition Although a detailed report of these results is beyond the scope of the present paper [see (7)] we believe the following summary of these results will be of interest to the reader

In Cl^--free media the PD is inverted (nutrient side negative) and H^+ secretion is about 25% of that in Cl^- solutions A variety of agents or procedures which reduce the H^+ rate produce a concurrent reduction in the magnitude of the inverted PD—the H^+ rate goes to zero and the PD to about zero In other words the PD is a criterion for the presence of H^+ secretion Using the inverted PD as a criterion for H^+ secretion neither aminopyrine nor imidazole inhibit H^+ secretion i e they do not appreciably affect the magnitude of the inverted PD Addition of thiocyanate (15 mM) to the secretory solution of a Cl^--free preparation

resulted in the inverted PD going to about zero and a reduction of the H^+ rate to zero With thiocyanate in the secretory solution addition of either imidazole or aminopyrine to the nutrient side results in a rapid restoration of the inverted PD i e re-establishment of the H^+ secretory state

Discussion

Once we found that one weak base did not inhibit secretion but was simply neutralizing the secreted H^+ ions it was not surprising to find that another did also One could expect that any weak base with the proper pK_a which readily diffused across the gastric mucosa should give essentially the same results

The finding that both weak bases would reverse thiocyanate inhibition of H^+ secretion was unexpected and surprising The fact that the H^+ rate was restored without much change in PD and resistance is challenging A study of the effects of other weak bases might help us to determine the mechanism of this reversal and/or the properties of the compounds which are responsible for this most unusual action

The results with nitrite and thiocyanate raise the question as to whether the mechanism of action of these two inhibitors is the same

The foregoing results should lead to a better understanding of thiocyanate inhibition of H^+ secretion which in turn should throw considerable light on the basic mechanism of H^+ production

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Factors in the Development of Experimental Ulcers

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ABSTRACT Clo ed cs of bullfrog stomach ulcerated in the absence of exogenous CO_2 but only when HCO_3 was absent from the nutrient s lution Open heets of stripped bullfrog mucosa bathed in 5 mM PO_4 buffer nutrient solution and gassed with 100% O_2 on N and 100% N_2 on S showed much more profound decreases in P D Isc and R when exposed to a mucosal pH of 2 than identically treated tissues with a nutrient Ringer s solution In 95% O_2 5% CO_2 histamine stimulated frog mucosa withstood an imposed secretory pH of 2 25 for 6 hours or 120 mM HCl for 30 minutes far better than burimamide-inhibited mucosae In vivo experiments in lightly anes thetized rabbit howed that resting stomachs (10 of 10) xposed t 120 mM luminal ac d uniformly ulcerated wherea only 1 of 10 tomachs timulated with histamine ulcerated Measurement of the pH of th lamina propri with ntimony microelectrodes during the latter experiments howed that the pH in th resting tissues decreased from 7 3 _ 0 1 t 6 9 _ 0 1 one hour aft r exposure to the luminal acid while there was no significant change in the pH in th hi tamine treated rabbits

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Davi and coworkers (1 2) demonstrated that sacs of amphibi n ga tri mucos lmo t unif rmly ulcerated when treated with acet lamid or when CO_2 and HCO_3 were removed from th incubating med um Thes investigat rs suggested that the absence of CO_2 aused sufficient lkalinization of the tissu to produce ulceration The pre ent tudies were done t elucidate further not only the mechanism of ul rat on in the absence of CO_2 but also to g in further ns ght int the rol of back diffusion of H^+ in the pathogenesis f ul e

Materials and Methods

In the experiments on amphibian tissue the gastric mucosa from Rana catesbeiana was used in eith r closed tubes or open sheets The sacs of mucosa were made by removing the entire coat of muscularis propria while maintaining the mucosa as an intact tubular structure After rinsing the lumen with 120 mM NaCl the ends of the tube were ligated with silk to exclude duodenum and esophagus The tubes were gassed in a 20 ml bath with either 100% O_2 or 95% O_2 5% CO_2 for 6 hours using differing nutrient solutions (see below) after which the sacs were opened and inspected under a dissecting microscope Some of the tissues were fixed in 10% formalin and stained with hematoxylin and eosin

To simulate as nearly as possible conditions within the sacs paired open sheets of bullfrog mucosa taken from the same frog were mounted between the halves of two separate lucit chambers One half was gassed with 100% O_2 on both surfaces after mounting in an Ussing chamber while the other half mounted in a separate chamber was gassed with 100% O_2 on M and 100% N_2 on S Each set of tissues was exposed to a nutrient solution of either mM phosphat (pH of 7 4 at end of experiment) or standard amphibian Ringer s s lution containing 18 mM HCO_3 (pH 9 t end of experiment) After stabilization of the tissue for at least 30 minutes to ascertain viability and H secretion the pH of the secretory solution was changed from 120 mM NaCl to a pH of 2 0 by the ddition f concentrated HCl and the P D Isc and R were measured for 4 hours

In a separate set of studies open sheets of bullfrog mucosa were mounted de cribed bove but were gassed with 95% O_2 5% CO_2 on both sides The mucosal solution was 120 mM NaCl and the nutrient solution was amphibian Ringer s (18 mM HCO_3) with 10 mM glucose but without histamine In paired halves of tissue H s cretion was either complet ly inhib ted with burimamide 4 mM or the tissue was allowed t secret spontaneously In some experiments after inhibition had been chieved hi tamine 4 X 10 ^{4} M was added to the nutrient solution and H ecret on began gain After stabilizat on

for 60 minutes the secret ry solution was changed to and maintained at a pH of 2 25 for 3 1/2 hours r a solution of 120 mM HCl was kept on th secretory surface for 30 minutes The acid s cretory rat was measured using the pH stat t chnique (Radiometer Copenhagen Denmark) The transmucos l potential difference (P D) was measured between tw agar bridges (1 M NaCl in 4% gar) connected through c lomel ref rence electrodes to a voltmeter and a recorde A direct current was passed cross the tissue between two othe salt agar bridges in contact with Ag AgCl electrode The tissue lectrical resistance (R) was calculated from the change in P D at 0 5 seconds after passag of a 100 μ A current across the tissue Correction was made for the R of the bathing fluid (~45 ohms cm^2)

In lightly anesth ti ed rabbits the pH within the gastric wall was d t rmined with an antimony microelectrode placed in the lamina propria with a micromanipul tor afte a button f seros and musculari propria had been excised The lumen f th stomach was exposed to 150 mM NaCl during the control period nd 120 mM HCl 30 mM NaCl in the experimental period In half of th rabbits histamine (2 5 mgm/kilo/mi) was given intravenously t stimulate secretion

Results

In amphibian Ringer s (18 mM HCO_3) and 100% O_2 (final pH_N~9) only 1 of 10 sacs ulcerated whereas 8 of 10 sacs in 5 mM PO_4 buff and 100% O_2 (final pH_N~7 4) had ul ers (p < 0 01) In 100% O_2 6 of 10 sacs ulcerated in unbuffered nutrient salt (pH_N~8 5) 3 of 10 with 25 mM PO_4 buffer (final pH_N~6 3) and 4 f 10 with a different 25 mM PO_4 buff (final pH_N~7) had ulcers Eight of 8 acs gassed with 100% O_2 in nutri nt soluti n of HEPES buffe at pH 7 4 developed ulcers whereas only 5 f 8 ulcerated with HEPES buffer at pH 6 3 Only 1 of 10 contral ca in Ringe s and gassed ith 95% O_2 5% CO_2 ul erated

In the open heet experiments done to simulat th losed sacs the P D Isc and R f th tissues xposed to a nutrient solution

of amphibian Ringer s solution showed insignificant changes wh ther gassed on both sides with 100% O_2 or in 100% O_2 on N with 100% N_2 on S In sharp contrast those mucosae in 5 mM PO_4 nutrient solution showed highly significant decreas s in P D Isc and R which wer especially marked when the secretory solution was gassed with N_2 (Figures 1 nd 2) None of the open sheet tissues ulcerated

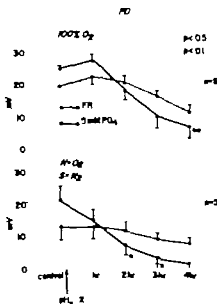


Figure 1 Changes in P D in isolated frog mucosa exposed either t 100% O_2 in N and S or 100% O_2 in N and 100% N_2 in S Values shown were obtained after initial stabilization with 120 mM NaCl on S and either 5 mM PO_4 buffer or amphibian Ringer s (18 mM HCO_3) on N At point shown by arrow S was changed to pH 2 0

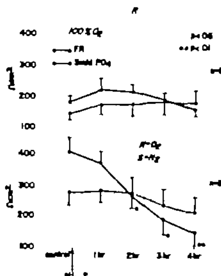


Figure 2 Changes in R is is lated frog mucosae exposed either to 100% O_2 in N and S r 100% O_2 in N and 100% N_2 in S Values hown were obtained after initial stabilization with 120 mM NaCl on S and eithe 5 mM PO_4 buffer o amphibian Ringer s (18 mM HCO_3) on N At point shown S was changed t pH 2 0

Exposure f the open sheets of frog mucos to a ecret ry pH f 2 25 for 3 1/2 hours und r otherwise standard conditions caused a highly significant drop in the P D (30 _ 2 6 mV to 12 1 _ 3 1 mV) and Isc (70 _ 9 2 to 22 4 _ 5 6 M amps/cm^2) of those ti ues inhibited with burimamide whereas no change in these measurements occurred in ither the spontaneously secreting or histamine stimulated tissue None f the mucosae showed a significant chang in R In the flat heets exposed to 120 mM HCl for 30 minutes the spontaneously secreting tissues showed a small rise in P D and Isc without a change in R whil the H^+ secretion decreased slightly from 4 5 _ 0 4 to 3 9 _ 0 5 M Eq/cm^2/hr The burimamid inhibited ti sues however showed tatistically highly significant decrements in P D (ΔPD=8 1 _ 9 mV) Is (ΔIsc 24 1 _ 3 2 M amps/cm^2) and R (ΔR=63 2 _ 16 4 Ohms cm^2) I none of these open sheet experi ments did the t ssue ulcerat

Exposure of the rabbit mucosa in vivo to 120 mM HCl for one hour was followed by ulceration of the fundic mucosa in 10 of 10 animals in the resting state whereas only 1 of 10 stomachs similarly exposed to 120 mM HCl ulcerated during stimulation with histamine The pH of the lamina propria in 10 rabbits in the resting state decreased from 7 3 _ 0 1 to 6 9 _ 0 1 one hour after exposure to the acid while there was no significant change in the pH of the lamina propria of the histamine treated animals

Discussion

The experiments with the sacs of gastric mucosa suggest strongly that the removal of CO_2 alone does not account for the ulceration which develops in the sacs Rather it appears that it is the absence of nutrient HCO_3 which is the common denominator in the ulcerated tissues

Ther is little doubt that removal of CO_2 from amphibian gastric mucosa causes a rapid rise in the pH of the tissue (3) However in the sac experiments of Davies and his coworkers (1 2) nly tho e tissues which secreted at vigorous rates ulcerated while thos which secreted poorly or not at all did not develop ulcers These workers and Terner (4) also showed that back diffusion of secreted H and its appearance in the nutrient medium tended to occur in the most severely ulcerated sacs In addition Werther et 1 showed that ulceration of the canine gastric mucosa after acetazolamide was related to an acceleration by the drug of luminal loss of H (5) It would appear therefore that it was the accumulation of H in the luminal content which was responsible for the ulceration of the ti sue within the sacs The presence of HCO_3 in the nutrient solution may enabl a tissue to withstand a greater load of luminal H since Hersey (3) has shown that alkalinization f th nut ent solution with HCO_3 causes a very clear rise in ntra llul r pH

Our data l arly nd c t that a mucosa which i not vigorously secreting whethe n tro (frog muc sa treated with burimamide)

or in vivo (rabbit in the resting state) tolerates luminal acid very much less well than the histamine stimulated tissue In support of the fi ding of lectrical d terioration of inhibited tissues in vitro is the invariable ulceration found in the in vivo rabbit in the resting stat W believe that th marked decrease in measured pH of the lamina propria in the resting rabbit mucosa is th first clear demonstration in vivo of ba k diffusion of luminal H

Our concept is that the gastri mucosa t lerates changes in intracellular pH but within limits (6 7 8 9) If the tissue becomes relatively cidi because of inhibition of secretion as shown by Hersey (3) or ourselves (6) then luminal cidificati or even acidification on the nutrient urface (10) becomes xtremely detrimental Wh ther mucosa ulcerates then will depend on its acid base balance which t least to ome extent influenced by the secretory t te of the tissue In additi n the mucosal blood flow probably plays n important role in the ability of th mocosa t tolerat luminal H in view of our own result in thes studies with histamine stimulated rabbit mucosa nd the res lts of Moody et 1 (11) reported at this meeting The orrelation of the presen or absence of ulceration in th rabbit experiments with changes in pH in the lamina propria sugge ts strongly that back diffusion of luminal H has indeed been demonstrated in vivo and that the ability f the mucosa to dispos of back-diffused H whether by alkaline tide or increased mucosal blood flow is the most important factor in the path genesis f ulc r

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Micropuncture Studies Using the Amphibian Fundic Gastric Mucosa, in Vitro

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ABSTRACT: Microelectrodes were used to measure the membrane potential from the surface or oxyntic cells of amphibian gastric mucosa in vitro; also recorded were transmucosal electrical resistance and ratio of cell membrane resistances Mecholyl added to a Necturus mucosa not secreting acid produced an effect on the surface cell that could be explained by increasing the E_{Cl^-} on the nutrient membrane The effects of mecholyl on the oxyntic cells could be explained by an increase in E_{Cl^-} on the secretory membrane These effects could be blocked by atropine and were not observed in Cl^- free solutions The effects of SCN^- on Necturus not secreting acid could be explained by changes in E_{Cl^-} on the secretory membrane of the oxyntic cell In the Rana pipiens SCN^- effects could be explained by a reduction in E_{H^+} on the secretory membrane of oxyntic cells

Introduction: The objective of the present study was to determine the location of the voltage or resistance changes in the gastric mucosa When using microelectrodes to estimate intracellular potentials or transmembrane resistance it was desirable to establish experimental conditions in which the parameters change at a fast rate i e 5 to 20 mV/min or 25 to 100 cm^2/min Several conditions met these criteria: 1) Mecholyl 10^{-6}M added to the nutrient (serosal) fluid of non-acid secreting Necturus 2) SCN^- 20 mM added to the secretory (lumen) solution of a Rana pipiens gastric mucosa that was secreting acid These conditions will be explained later

Several problems became apparent when micropuncture techniques were applied to the study of the electrophysiology of the stomach: multiple cell types and multiple cell layers;

spontaneous motility produced by the smooth muscles in the submucosa; mucous coat covering the surface of the lumen (greatly reduced visibility); measurement of a junction potential when the microelectrode tip was in the lamina propria Even with these problems this technique produced data that could not be obtained with other procedures and the information can add to our understanding the mechanisms of acid secretion

Methods: The animals were obtained weekly when available from commercial sources; the Rana pipiens were not available during the winter of 1977 and Necturus maculosus were used during that time period The frogs were housed as previously described (1) and the Necturus were stored in distilled water tanks oxygenated and maintained at 18 C The animals were fed fish food The Necturus were maintained for at least three days at 23 C before being used in experiments

The gastric mucosa was mounted in an in vitro chamber 1 23 cm^2 active area and bathed in the following: Nutrient solution (in mM/l) - 91 Na^+ 5 K^+ 1 5 Mg^{++} 1 0 Ca^{++} 75 Cl^- 25 HCO_3 5 glucose; Secretory solution (in mM/l) 91 Na^+ 5 K^+ 75 Cl^- 10 5 SO_4^{--} 5 glucose 10 sucrose The solutions were oxygenated and circulated by 95% O_2 and 5% CO_2 The experimental setup was described previously (2) The following parameters were measured simultaneously - V_{MN} V_{MS} V_{SN} R_T and R_S/R_N M = Microelectrode N = Nutrient S = Secretory R_T = transmucosal resistance R_S/R_N = ratio of membrane resistances V_{MN} indicated that the voltage was recorded from microelectrode in reference to the nutrient solution i e the nutrient solution served as the ground or reference point

Microelectrodes (M E) were pulled by use of a two-stage puller and filled with 3 M KCl; the average tip resistance of the microelectrodes was 18 Meg Ω Differential amplifiers (W P Instrument Co #750) were used and the outputs recorded on a four channel potentiometric paper recorder The micropunctures were visualized by use of a dissecting microscope 40 to 120 X magnification using a 15 mm working

distance objective The criteria for a good puncture were as follows: less than 20% change in M E tip resistance during or after a puncture compared to the control; the voltages should return to control values after withdrawal of the M E tip Each value reported was from experiments in which the M E tip was in the same cell for the control and test period

Techniques for micropuncture of a surface cell - The microelectrode was attached to a 3 M KCl agar bridge that made contact with 3 M KCl and a calomel electrode was connected to the amplifier The movement of the micro-electrode was controlled by a micromanipulator and the tip of the electrode was viewed under the microscope With the tip of the M E in the secretory solution the V_{MN} equalled V_{SN} and V_{MS} was zero The M E tip was lowered under visualization and a cell was punctured and a simultaneous negative change recorded for V_{MN} and V_{MS}

Techniques for micropuncture of oxyntic cells - (1) Histological sections of Necturus and Rana pipiens gastric mucosa were studied and the average depth of the oxyntic cells from the surface was estimated using a micrometer in the eyepiece of the microscope The average depth was 200 µ (range 100-300 µ depth) Microelectrodes were pulled; and with a micromanipulator and a catheter black acrylic dye thinned with acetone was injected to mark a spot 200 µ from the tip of the M E on the taper of the electrode This spot was approximately 10 µ in diameter and was visible as the M E tip was lowered to 200 µ depth in the tissue Since at this depth the microelectrode tip could be in an oxyntic cell or in the lamina propria other techniques were tried (2) Another approach was to use double barrelled M E One lumen of the M E was filled with 3 M KCl and used for the regular voltage recordings; a more stable reading was obtained than when the voltage was recorded from an electrode filled with dye The other lumen was filled with 2% Niagara Sky Blue 6B (C I 24410 #R754 Baker Chem Co) After a puncture the recordings were made with the 3 M KCl side;

the amplifier was shorted and current was injected through the side containing the dye To facilitate this procedure another technique was added: a blunt electrode (10 μ tip diameter) was filled with lithium carmine (red dye) and two red spots were injected into the mucosa separated by a distance of about 10 μ These red spots served as visual markers for the blue dye injections and aided in the handling of the frozen tissue After an experiment the tissue was removed from the chamber frozen and sectioned by use of a cryostat and the spots were located (3) Still another procedure was tried that was much easier and less tedious Recordings from V_{MN} and V_{SN} were amplified 4X to increase the sensitivity of R_T and R_S/R_N The microelectrode tips were marked as described above with a paint spot at 200 μ from the tip; the tip was lowered and a change in R_S/R_N was observed When the M E tip was in an oxyntic cell there was a measurable ratio of R_S/R_N; this was not observed when the M E tip was in the lamina propria To measure the junction potential when the M E tip was in the lamina propria two different types of experiments were performed: (a) the mucosa was scrapped and the M E tip was lowered to puncture an area free of cells (b) the mucosa positions was reversed so as to place the submucosa upward and the M E punctured from the submucosal side These two types of experiments produced generally the same results i e average value of V_{MN} = -8 6 mV in 35 punctures

Results: Experiments using Necturus

Mecholyl effects— It has been shown previously that mecholyl produced a transient change in the transmucosal potential (V_{SM}) and a reduction in the resistance (3 4) but the effects on the oxyntic cell had not been reported Mecholyl (B methylacetyl choline) 10^{-6}M was added to the nutrient solution of mucosae not secreting acid and the effects are shown in Table 1 Simultaneous experiments were performed using the pH stat technique and these animals were not secreting acid The changes due to mecholyl could be blocked by atropine 10^{-4}M or by replacing all the Cl^- in the

two bathing solutions with SO_4^{--} Once a change in V_{SN} of approximately 8 mV was obtained then changing the solutions and adding mecholyl again produced no response (5)

Table 1

Location of M Electrode Tip	Condition	V_{MN} (mV)	V_{MS} (mV)	V_{SN} (mV)	R_T (Ω cm^2)	RS/RN	n
	Control	−17 1	10 2	−27 3	770	8 4	7
Oxyntic	Mecholyl	−16 7	16 0	−32 7 *	560	6 4	7
Cell	Δ[1]	0 4	5 8	−5 4	−210	−2 0	–
	± SEM[2]	1 4	3 5	1 5	65	0 7	–
	Control	−52 8	−28 7	−24 1	855	5 6	11
Surface	Mecholyl	−56 7	−28 0	−28 7	575	4 6	11
Cell	Δ[1]	−3 9	0 7	−4 6	−280	−1 0	–
	± SEM[2]	1 1	1 0	4 0	65	0 4	–

[1] Mecholyl − control values; [2] ± SEM of Δ values
* Significance at the 5% level; Significance at the 1% level

Experiments using Necturus and testing the effect of Mecholyl 10^{-6}M in the nutrient solution The various Δ values were tested using the t test (null hypothesis)

SCN^- effects on Necturus—When SCN^- was added to Necturus gastric mucosae not secreting acid the results are summarized in Table 2 Note that the change in V_{SN} is in the opposite direction to that normally observed using Rana pipiens (6) Experiments using Rana pipiens Several laboratories over the past few years reported the effects of SCN^- on amphibian gastric mucosa and this led me to try to determine the site of action of SCN^- (7 8 9) Table 3 shows a summary of the results in which 20 mM SCN^- was added to the secretory solution of Rana pipiens secreting acid

In all of these experiments the microelectrode was in a surface cell or an oxyntic cell for the control and test periods

Table 2

Location of M Electrode Tip	Condition	V_{MN} (mV)	V_{MS} (mV)	V_{SN} (mV)	R_T ($\Omega\ cm^2$)	RS/RN	n
Oxyntic Cell	Control	-20 5	12 0	-32 5	930	9 7	6
	SCN^-	-19 8	8 5	-28 3	940	9 8	6
	Δ^1	0 7	-3 5	4 2	10	0 1	-
	$\pm$ SEM^2	0 5	1 3	1 5	8	0 2	-
Surface Cell	Control	-53 7	-24 9	-28 8	790	4 8	15
	SCN^-	-53 2	-28 9	-24 3	770	4 8	15
	Δ^1	0 5	-4 0	4 5	-20	0	-
	$\pm$ SEM^2	1 0	2 2	1 6	25	0 2	0

[1] SCN^- - control values; [2] $\pm$ SEM of Δ values;
*Significance at the 5% level; *Significance at the 1% level

Experiments using the Necturus and testing the effects of SCN^- 20 mM in the secretory solution Histamine 10^{-4} M was in the nutrient solution

Table 3

Location of M Electrode Tip	Condition	V_{MN} (mV)	V_{MS} (mV)	V_{SN} (mV)	R_T ($\Omega\ cm^2$)	RS/RN	n
Oxyntic Cell	Control	-14 0	17 4	-31 4	210	6 4	7
	SCN^-	-12 7	28 9	-41 6 *	230	6 3	7
	Δ^1	1 3	11 3	-10 2	20	-0 1	-
	$\pm$ SEM^2	1 5	3 9	1 7	4	0 4	-
Surface Cell	Control	-50 6	-19 8	-30 8	215	5 6	6
	SCN^-	-50 8	-10 0	-40 8*	235*	5 8	6
	Δ^1	-0 2	9 8	-10 0	20	0 2	-
	$\pm$ SEM^2	1 8	2 1	2 0	15	0 2	-

[1] SCN^- - control values; [2] $\pm$ SEM of Δ values;
*Significance at the 5% level; Significance at the 1% level

Experiments using the Rana pipiens and testing the effect of SCN^- 20 mM in the secretory solution Histamine 10^{-4} M was in the nutrient solution

Discussion: The mecholyl effect on the surface cell indicated that this cell type was not completely passive The R_S/R_N recorded for control vs mecholyl averaged 5 1 and the V_{MS}/V_{MN} was 0 2; these are not equal Therefore the change in V_{MS} or V_{MN} could not be due to a voltage change produced by a change in current flow from the oxyntic cell i e this was assuming the surface cell acted as a passive shunt for current generated by the oxyntic cell and this was not the case under these conditions Mecholyl does not produce an observable effect using Rana pipiens

The problem of determining when the microelectrode tip was in an oxyntic cell was a difficult one but the depth measurement and resistance ratio method gave a fairly accurate and rapid method With a possible junction potential of -8 6 mV V_{MN} between 0 and -10 mV was not used; this restraint could put a bias on the values taken for the record

The blocking effect of atropine suggests that the mecholyl was mediated by a cholinergic pathway The fact that the mecholyl effect was not observed in Cl^- free media indicates that the response was due to a change in Cl^- transport or Cl^- permeability

With the Necturus not secreting acid the addition of SCN^- resulted in a change in V_{SM} becoming more positive; normally with the addition of SCN^- to a Rana pipiens mucosa secreting acid the P D became more negative With no H^+ secretion but with Cl^- transport the Cl^- EMF was influencing the secretory membrane of the oxyntic cell These results indicated that SCN^- reduced the E_{Cl^-} on the secretory membrane of the oxyntic cell in non-acid secreting Necturus The results using Rana pipiens were as would be predicted assuming a separate site for H^+ and Cl^- transport for the effects of SCN^- on the E_{H^+} located on the secretory membrane of the oxyntic cell

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Stimulation and Inhibition of Acid Secretion in the Isolated Guinea Pig Gastric Mucosa

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A method to study pharmacological effects of various substances on acid secretion from the isolated guinea pig gastric mucosa has been developed (1)

Male and female guinea pigs have been used After sacrificing the animals the stomach is rapidly taken out and pieces of the acid secretory portion are mounted on the end of plastic funnels The outer muscular layer is carefully stripped off The mucosal preparation is then immersed into an organ bath containing a buffered nutrient solution Compounds tested are added to this medium To the mucosal side is added a non-buffered solution allowing continuous pH-determination and titration

By adding various compounds to the nutrient solution their effect on acid secretion from the mucosal preparation can be studied

In a first series histamine was added in gradually increasing doses and cumulative dose-response curves were constructed (1) By pretreatment with an H_2-receptor blocking agent metiamide the dose-response curves for histamine were displaced to the right in a parallel manner without affecting the maximal histamine response Calculations of the dissociation constant have shown that metiamide and histamine interact by a competitive mechanism in the present preparation

In another series of experiments the mucosae were pretreated with a substituted benzimidazole (H 83/88) which has been found to be a potent inhibitor of histamine stimulated

acid secretion in vivo (to be published) Following administration of gradually increasing doses of histamine no parallel displacement was observed but a significant dose-dependent depression of the maximal histamine in four experiments The data suggest that H 83/88 inhibits histamine stimulated acid secretion by a non-competitive mechanism and thus showing an antisecretory pattern different from that of H_2-receptor antagonists

Following administration of the dibuturylic form of cAMP to the nutrient medium a small stimulatory effect was observed However after pretreatment with theophylline (10^{-3}M) which by itself did not affect the secretory rate the acid response to db-cAMP was significantly increased (Fig 1)

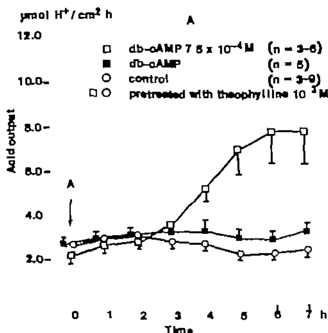


Fig 1 The effect of dibuturyl-cAMP before and after pretreatment with theophylline on acid secretion from the isolated guinea pig gastric mucosa

The present data suggest that db-cAMP is a stimulator of acid secretion from the isolated guinea pig mucosa which is in agreement with data from other species (2 3 4)

In another study it was found that the acid response to histamine was increased following theophylline treatment (Fig 2) Our results are in agreement with the findings in similar experiments on the isolated rabbit mucosa (3) These data indicate that cAMP is a link between the histamine receptor and the H^+-output and that inhibition of phosphodiesterases by theophylline facilitates the secretory mechanism activated by low amounts of intracellular nucleotide

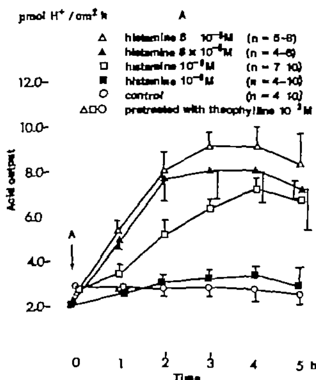


Fig 2 The effect of histamine before and after pretreatment with theophylline on acid secretion from the isolated guinea pig gastric mucosa

In a preliminary study acid secretion was stimulated by db-cAMP (10^{-3}M) following theophylline pretreatment Administration of metiamide (10^{-5}M) did not change the acid response to db-cAMP in agreement with the concepts that metiamide interferes with H_2-receptors presumably localized on the outside of the parietal cell and that db-cAMP stimulates acid secretion by an intracellular mechanism

After administration of the substituted benzimidazole (H 83/88) (10^{-5}M) a decline in the acid secretory response to db-cAMP was observed (Fig 3) The present data may indicate that H 83/88 exerts its inhibitory action at a site peripheral to the H_2-receptor and at a later stage than the cAMP step in the parietal cell

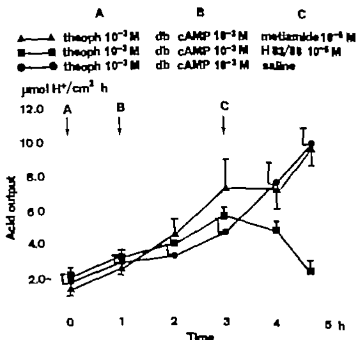


Fig 3 The effects of metiamide and H 83/88 on dibuturyl-cAMP induced acid secretion from the isolated guinea pig gastric mucosa

The present technique may be of value for studying the interaction between various physiological and pharmacological stimuli and inhibitors

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Cellular Mechanisms of Gastric Hypersecretion in Pylorus-ligated Rats

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ABSTRACT: The effects of epinephrine theophylline Degranol[R] actinomycin D tetracycline, ATP, ADP, AMP cyclic 2 3 -AMP cyclic 3 5 -AMP (cAMP) have been studied on the gastric secretory responses of pylorus-ligated rats; on the other hand the tissue levels of mucosa ATP, ADP, AMP cAMP lactate and pyruvate were measured It has been found: 1 the gastric hypersecretion of pylorus-ligated rats could be inhibited dose-dependently by epinephrine theophylline, Degranol[R] actinomycin D tetracycline, ADP, AMP cyclic 2 3'-AMP and cAMP; 2 the plasma level of immunoreactive gastrin did not change in different times (2 4 7 and 16 hours) after pylorus-ligation; 3 the tissue contents of ATP cAMP and lactate significantly decreased in 2 4 hours after pylorus-ligation; while the tissue levels of ADP and AMP significantly increased in that time; 4 the increased tissue level of mucosa ADP in 4 hours after pylorus-ligation could be prevented by atropine epinephrine atropine + theophylline epinephrine + theophylline histamine; 5 the decreased mucosal level of cAMP in 4 hours after pylorus-ligation could be elevated by theophylline epinephrine atropine + theophylline epinephrine + theophylline but not by histamine and histamine + theophylline It has been concluded: 1 the increase of RNA synthesis depending on DNA DNA and protein translation to be needed for the development of gastric hypersecretion; 2 the gastric hypersecretion of pylorus-ligated rats is an ATP-dependent process; 3 the development of gastric hypersecretion associates with an increase of ATP transformation to ADP and with an decrease of ATP transformation to cAMP; while the blockage of gastric hypersecretion associates with a decrease of ATP transformation to ADP and with an increase of ATP transformation to cAMP; 4 an extra- and intracellular feed-back mechanism system exists between the ATP-ADP (membrane ATP-ase) system and ATP-cAMP (adenylate cyclase) system of gastric mucosa in time of development of gastric hypersecretion

The gastric hypersecretion of rats is developed by

pylorus ligation (1 2 3) This model is widely used method by pharmacologists and gastroenterologists to study the mechanism of gastric hypersecretion and its inhibition This model has been used by our work-team to approach the gastric secretory mechanisms and ulcer development (3-7)

In this paper the cellular mechanisms of gastric hypersecretion have been studied in pylorus-ligated rats The aim of this study was to approach the biochemical background of gastric hypersecretion in pylorus-ligated rats

MATERIALS and METHODS

The observations were carried out on both sexes of Wistar strain rats weighed 180 to 210 g The animals were fasted with water ad libitum for 24 hours before the examinations The pylorus-ligation was carried out under the light ether anesthesia Each animal received 6 ml physiological saline solution immediately after the surgery to prevent the exsiccosis of animals

Different pharmacological and biochemical examinations were carried out:

1 dose-response curves were determined for epinephrine theophylline actinomycin D Degranol[R] tetracycline, ATP, ADP AMP cyclic 2 3 -AMP and cAMP; on the other hand the effects of atropine epinephrine and their different combinations with theophylline were studied on the gastric secretory responses in pylorus-ligated rats;

2 the following biochemical examinations were carried out: determination of tissue levels of ATP ADP AMP cAMP lactate and pyruvate in the fundic gastric mucosa The tissue level of ATP, ADP, AMP lactate and pyruvate was measured by enzymatic way (Biochemica test Combination Boehringer Mannheim GMBH) The mucosal cAMP was determined by radioimmunoassay (Schwarz/Mann USA) The protein content was assayed by biuret reaction (8) The substrate levels were calculated in accordance to 1 0 mg mucosal protein;

3 the plasma level of immunoreactive gastrin was measured by radioimmunoassay (CEA-IRE-SORIN)

The animals in the different experimental groups were killed at different times (1 2 4 5 7 16 17 and 24 hours) after pylorus-ligation The gastric volume was measured the titratable acidity was determined by titration with 0 10 N NaOH to pH 7 0 using a pH-titrimeter(Radelkis Budapest) The volume of gastric secretion was expressed in ml / 100 g body weight; the H^+ output in µEq/100 g body weight The H^+ concentration was expressed in mEq/liter

The results are presented as means ± SEM The statistical analysis between the two groups was carried out by Student's t test; while the correlation analysis according to Fisher(9)

The following drugs were used for the experiments:actinomycin D(Chinoin Budapest) atropine(atropinum sulfuricum Biogal Debrecen) cAMP(Sigma Chemical Co St Louis) ,ADP(Reanal Budapest) ,ATP(Reanal Budapest) AMP(Reanal Budapest) cyclic 2 3 -AMP(Reanal Budapest) Degranol^R(1 6-bis-/2-chlorethylamino/-1 6-didesoxy-D-mannitol)(Chinoin Budapest) epinephrine(Tonogen^R Richter Gedeon Budapest) histamine (histamine dihydrochloricum Peremin^R Chinoin Budapest) tetracycline (Tetran B^R Chinoin Budapest)

RESULTS

The volume of gastric secretion proportionally increases with the time after pylorus ligation; the H^+ output reaches its peak at 7 hour while the H^+ concentration decreases after 7 hr followed the pyloric occlusion (Fig 1) The time period signaled with arrow is an excellent one to study the developmental mechanisms of gastric hypersecretion and the possible ways to inhibit them

The gastric hypersecretion of pylorus-ligated rats can be inhibited dose-dependently by actinomycin D (40 400 800 and 1000 µg/kg) Degranol^R (in doses of 0 4 4 40 and 100 mg/kg) tetracycline (in doses of 4 and 40 mg/kg) epinephrine (in doses of 0 1; 0 4 and 1 0 mg/kg) and theophylline (in doses of 0 1 ; 0 2 and 1 1 mg/kg)

The tissue level of mucosal cAMP significantly decreased

at 2 hr after pylorus ligation which proceeded in time the significant increase of gastric hypersecretion (Fig 2)

The plasma level of immunoreactive gastrin remained at the same level before and after Shay-periods (Fig 3)

The mucosal levels of ATP, ADP, AMP cAMP lactate and pyruvate were measured in time of first period (0 to 4 hr) after pylorus ligation The mucosal level of ATP cAMP and lactate significantly decreased ($p < 0\ 001$; n=26); mean-

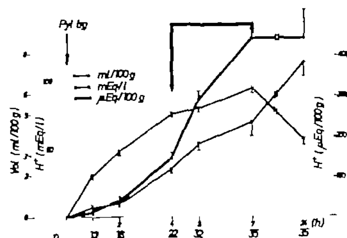


Fig 1 Secretory pattern of pylorus-ligated rats The results are given as means ± SEM n indicates the number of animals the abcissa shows the time after pylorus-ligation

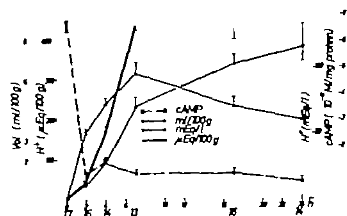


Fig 2 Correlation between the gastric secretory responses and the mucosal level of cAMP /expressed in pmoles per mg mucosal protein/ The abbreviations are the same as in Fig 1

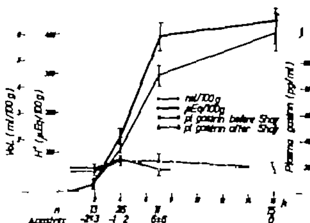


Fig 3 Correlation between the gastric secretory responses and the plasma level of immunoreactive gastrin of pylorus-ligated rats The abbreviations are the same as in Fig 1

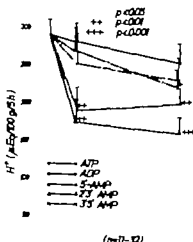


Fig 4 The effects of adenine nucleotides (ATP ADP cAMP cyclic 2 3 -AMP and AMP) on the gastric H output of pylorus-ligated rats The abbreviations are the same as in Fig 1

while the tissue level of ADP and AMP significantly increased ($p < 0\ 001$; n=26) in that time When the correlation was analyzed between the mucosal ATP and gastric H^+ output then

a negative and mathematically significant correlation was obtained ($r = -0\ 578$; $p < 0\ 001$; $n = 38$; regression line: $Y = 121\ 36 - 0\ 11\ x$)

The effects of theophylline atropine atropine + theophylline epinephrine epinephrine + theophylline histamine and histamine + theophylline were studied on the gastric H^+ output mucosal levels of ADP cAMP,AMP (Table I) The gastric H^+ output could be inhibited by theophylline atropine atropine + theophylline epinephrine epinephrine + theophylline The H^+ output was significantly increased by histamine The transformation of ATP to ADP was significantly inhibited by application of all drugs The mucosal level of cAMP could be elevated by application of theophylline epinephrine and epinephrine + theophylline No elevation of mucosal cAMP was produced by histamine and histamine + theophylline.AMP level could be significantly decreased by application of theophylline atropine + theophylline epinephrine + theophylline and histamine

The correlation analysis was carried out between the gastric H^+ output and the tissue level of mucosal cAMP on the other hand between the gastric H^+ output and the decrease of mucosal cAMP (deviation from the sham-operated group) at 2 4 and 7 hr after pylorus-ligation These correlations were found to be not significant between the examined parameters in all times

The effects of adenine nucleotides were studied on the gastric H^+ output in pylorus-ligated rats.A dose-dependent inhibition was caused by all adenine nucleotides (except of ATP) on the H^+ output When the cAMP was together applied with a large dose of theophylline (1 1 mg /kg) then its inhibitory effect remained the same as it was caused by cAMP alone

DISCUSSION

The punctual mechanisms of gastric hypersecretion are unknown in pylorus-ligated rats Some details of the suggested mechanisms (general biochemistry glycoproteins Mg^{2} $-Na^+-$

Table I The effects of theophylline (0 22 mg/kg) atropine (1 0 mg/kg) atropine (1 0 mg/kg) + theophylline (0 22 mg/kg) epinephrine (0 4 mg/kg) epinephrine (0 4 mg/ kg) + theophylline (0 22 mg/kg) histamine (20 0 mg/kg) histamine (20 0 mg / kg) + theophylline (0 22 mg/kg) on the gastric H^+ output tissue levels of ADP AMP and cAMP in the gastric mucosa of pylorus-ligated rats The gastric H^+ output (in µEq / 100 g body weight / 4 hr) ADP AMP (in nmoles / mg mucosal protein) and cAMP (in pmoles / mg mucosal protein) are expressed as means ± SEM n indicates the number of animals The p values were calculated in comparison with the results obtained in the Shay rats (without drug)

Groups of animals	H^+ output	ADP	cAMP	AMP
Sham operated rats (n 12)	-	13 ± 1	6 72 ± 0 37	4 ±0 5
Shay rats (without drug) (n = 14)	167 ± 30	144 _ 10	1 32 ± 0 06	14 ± 2
Shay + theophylline (n 14)	93 ± 14	36 ± 3 6+++	2 19 ± 0 04+++	5 1 ± 0 4+++
Shay atropine (n = 14)	28 ± 9 8++	21 ± 2 3+++	0 83 ± 0 03+++	18 ± 0 8
Shay + atropine + theoph (n 14)	13 5 ± 13 +	12 ± 1 8+++	1 09 ± 0 13+++	8 4 ± 0 6+ +
Shay + epinephrine (n = 14)	84 ±3 4+++	19 ± 0 7+ +	2 44 ± 0 09+++	11 ± 1 6
Shay epinephrine + theoph (n = 12)	24 ± 1 7+++	9 ± 1 3 ++	2 67 ± 0 15+++	5 ± 0 5+++
Shay + histamine (n = 15)	420 ± 55++	12 5 _1+++	0 53 ± 0 03+ +	7 7 ± 0 2+++
Shay + histamine + theoph (n 11)	225 ± 40	no tested	0 61 ± 0 03+++	no tested

= $p < 0\ 05$ + = $0\ 01 > p > 0\ 001$ + = $p < 0\ 001$

K^+-dependent ATP-ase) have been studied in our prior works (3-6 10)

The drugs used for the present observations have different attack-places on the cells: actinomycin D inhibits the RNA synthesis depending on DNA Degranol[R] inhibits the d e n o v o synthesis of DNA while the transformation of proteins can be inhibited by tetracycline (see ref 11) The gastric secretory responses - in pylorus-ligated rats - can be inhibited by these drugs indicating that the increase of RNA synthesis depending on DNA DNA and translation is needed to development of gastric H^+ secretion in this hypersecretoric model These results are in a good agreement with others previously published (3 4 12)

The development of gastric hypersecretion in pylorus-ligated rats is an ATP-dependent process which does not involve any increase of lactate level in the gastric mucosa

The breakdown of ATP - at the level of cytoplasma membrane - is present in two directions: transformation of ATP to ADP by membrane ATP-ase and 2 transformation of ATP to cAMP by adenylate cyclase When the products of ATP breakdown were measured then these ways were approached by the experiments (Table I) The essential role of gastric mucosa Mg^{2+}-Na^+-K^+-dependent ATP-ase has been proven in pylorus-ligated rats (7 10) The production of membrane ATP-ase activity was measured as mucosal ADP The transformation of ATP to ADP by the membrane ATP-ase can be inhibited by atropine epinephrine theophylline histamine and by different combinations of above mentioned drugs and by surgical vagotomy (13) The transformation of ATP to cAMP can be stimulated by epinephrine theophylline probably by atropine (but the cAMP is transformed further to AMP in the time of atropine effect); but it cannot be stimulated by histamine and histamine + theophylline Different conclusions have been drawn from these examinations: 1 the gastric H^+ secretion in the pylorus-ligated rats depends on the ATP breakdown in the gastric mucosa ; 2 the inhibition of

ATP transformation to ADP by atropine theophylline epinephrine and by combinations of atropine + theophylline and epinephrine + theophylline associates with the inhibition of gastric H secretion; 3 the ATP transformation to ADP results a significant inhibition on the ATP transformation to cAMP in pylorus-ligated rats; 4 the inhibition of ATP transformation to ADP by different drugs (epinephrine epinephrine + theophylline theophylline probably by atropine + theophylline) results an elevation of mucosal level of cAMP; 5 the increased level of mucosal cAMP represents an inhibition on the gastric secretion (in time of drug effects by epinephrine theophylline epinephrine + theophylline and probably atropine) ;6 the histamine effect does not depend on the transformation of ATP to ADP and on the ATP transformation to cAMP The following results suggest it:a the mucosal level of ADP remains the same in the time of histamine effect; b the mucosal level of cAMP does not elevate after histamine application (in time of increased H output) ; c theophylline does not stimulate the histamine effect on the gastric H^+ output; d the mucosal level of cAMP does not elevate by together application of histamine with theophylline

Suggesting some correlation between the mucosal cAMP system and the gastric H^+ secretion the following criteria are needed to be present between them:

1 time order (in both stimulation and inhibition) ;

2 mathematically closed correlation between the mucosal cAMP and the acid output;

3 mimicri effect

These criteria were evaluated in the pylorus-ligated rats:

1 the peak of acid output in pylorus-ligated rats is in 7 hr after pylorus occlusion The 4 hr was found to be suitable time to study the membrane ATP-ase system in order to physiological response (10) In that time the mucosal level of cAMPaextremely low in comparison with the sham-operated rats (Fig 2) The mucosal level of cAMP can be

elevated by different drugs (epinephrine theophylline epinephrine + theophylline) but its level never can reach the value obtained in the sham-operated rats It can be explained by that the ATP breakdown – in direction to ADP – represents an essential inhibition for the transformation of ATP to cAMP or by that the ATP can split to ADP because of the mucosal level of cAMP significantly decreased before;

2 no significant correlation was found to be present between the gastric H^+ output and the mucosal level of cAMP or between the gastric H^+ output and the decrease of mucosal cAMP at 2 4 7 hr after pylorus-ligation;

3 the cAMP has not a mimicri effect on the gastric H^+ output because the cAMP itself inhibits it; all adenine nucleotides (except of ATP) caused a dose-dependent inhibition on the gastric secretion as in Kidder s observations (14)

There are different arguments to suggest the inhibitory effect of cAMP on the gastric secretion in pylorus-ligated rats: 1 theophylline which increases the mucosal level of cAMP inhibits the gastric H^+ secretion 2 epinephrine which also elevates the level of mucosal cAMP inhibits the gastric H^+ secretion; 3 the mucosal level of cAMP significantly decreases before and in time of extremely increase of gastric H^+ secretion; 4 cAMP itself (and other adenine nucleotides) inhibits (inhibit) the gastric H^+ secretion; 5 the membrane ATP-ase system has an important role in the development of gastric hypersecretion and it can be inhibited by cAMP application (17) The suggestion for inhibitory effect of cAMP on the gastric H^+ secretion is in a good agreement with results by Thompson et al (18)

In our prior studies a feed-back mechanism system by different drugs and hormones has been suggested between the Na -K^+-dependent ATP-ase system and the adenylate cyclase system (19 20) It seems that this feed-back mecahnism system is present between the two – Na^+-K^+-dependent ATP-ase and adenylate cyclase – systems of the gastric mucosa in pylorus-ligated rats during the time (0 to 7 hr)

period after pylorus occlusion The experimental bases to suggest it are the following: 1 the gastric H^+ secretion of pylorus-ligated rats is an ATP-dependent process; 2 the transformation of ATP to ADP represents a significant inhibition for the transformation of ATP to cAMP; 3 the drug effects are contraregulatory ones on the two systems ; 4 the cAMP itself inhibits the gastric H^+ secretion; 5 the drugs elevating the mucosal level of cAMP – in the same time – inhibit the transformation of ATP to ADP (theophylline epinephrine epinephrine + theophylline atropine + theophylline); 6 the drugs inhibiting the gastric H^+ output can inhibit the membrane ATP-ase activity from the rat and human gastric mucosa

In the pylorus-ligated rats in which an extremely gastric hypersecretion is developed by pylorus ligation no elevation (or any significant change) of immunoreactive gastrin was observed in the plasma of rats (Fig 3) According to our opinion no gastrin response is in the development of gastric hypersecretion of pylorus-ligated rats These results are in a good agreement with our previously described observations: the gastric hypersecretion – in the pylorus-ligated rats – was found to be the same in presence or in absence of antrum (15 16)

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Interrelationships between the Cholinergic Influences, Gastric Mucosa Na^+–K^+-dependent ATPase, ATP, ADP, Ions of Gastric Juice and Basal Secretion in Patients

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ABSTRACT: The separations and measurements of the gastric mucosa Na^+-K^+-dependent ATP-ase, ATP, ADP were carried out in humans with different basal acid outputs (BAO); on the other hand the effects of acetylcholine parasympatholytics were studied on Na^+-K^+-dependent ATP-ase activity It has been found: 1 a positive and mathematically significant correlations was found to be present between a BAO values (2 40 ± 0 34 mEq/h) (means ± SEM) and the tissue contents of ATP in the gastric mucosa (472 ± 42 nmoles /mg DNA value) (r = 0 63; Y = 278 x – 190; n = 28; $p < 0$ 01); b BAO values (2 20 ± 0 37 mEq/h) and tissue contents of mucosa ADP (270 ± 19 nmoles/ mg DNA value) (r = 0 69; Y = 139 x – 36; n = 28; $p < 0$ 001); c tissue contents of ATP (426 ± 98) and ADP (246 ± 50) (r = 0 99; Y = 0 46 x – 60 ; n = 31 ; $p < 0$ 001) ; d gastric mucosa ATP (808 ± 182) and Na^+-K^+-dependent ATP-ase activity (2 60 ± 0 36 µmoles of P_i / mg membrane protein /h) (r = 0 66; Y = 0 001 x 1 94 ; n = 32 $p < 0$ 01) ; e BAO values and Na^+-K^+-dependent ATP-ase activity ($p < 0$ 001; n = 46) ; 2 the transformation of ATP to ADP by the way of membrane ATP-ase is open to acetylcholine effect while it is inhibited by parasympatholytics These results suggest a causal correlation between the gastric mucosa Na^+-K^+-dependent ATP-ase, ATP, ADP basal acid output and cholinergic influences in humans

The active transport of sodium and potassium ions across the cell membrane is functionally associates with a closely membrane-bound (Na^+-K^+-dependent or Na^+-K^+-activated) ATP-ase (1-3) This enzym can be inhibited by ouabain (4)

In animal observations the role of this enzyme system (cellular ATP-ADP) has been studied and its importance proven in order to relation of gastric H^+ secretion (5 6) The Na^+ K -dependent ATP-ase enzyme has been found to be

present in the human gastric mucosa (7-16) but its importance remained unknown from the point of gastric H^+ secretion and pharmacological regulation

The aim of this study was to examine the correlations between the gastric mucosa Na^+-K^+-dependent ATP-ase activity ATP ADP basal acid output and cholinergic influences

MATERIALS and METHODS

The observations were carried out in patients with peptic ulcer who underwent gastric resection because of peptic ulceration These patients have not received parasympatholytics under 10 days befo e their operation

The basal acid output was measured by a continuous suction of gastric juice for a three hours period and the average per one hour was taken to be the final result for BAO The gastric juice was titrated with 0 10 N NaOH to pH 7 0 using a pH-titrimeter indicating system (Radelkis Budapest) The gastric H^+ secretion was expressed as mEq/hour The gastric juice was analyzed for ion composition: the sodium potassium and calcium were measured phlamephotometrically the chloride according to Hamilton (17) The concentrations of ions were expressed in mEq/liter values

The adenine nucleotides were extracted from the human corpus mucosa by 0 6 M perchloric acid and separated from each other by an ion-exchange (Dowex I 200 to 400 mesh) chromatography (1 5 cm high in a 20 cm column of 0 7 cm diameter) ,ATP and ADP quantities were measured by UV spectophotometer (8 15 16) The tissue DNA was extracted by the method of Schmidt-Thannhauser (18) and its quantity measured by the sugar component (19) The tissue contents of mucosa ATP and ADP were expressed in nanomoles / mg DNA value

The membrane ATP-ase was prepared with differential centrifugation (20 000xg and 40 000xg) and treatment with 2 0 M NaI solution (7 11) The membrane ATP-ase activity was measured in an incubation system at 37°C by liberation of inorganic phosphorus (P_i) followed the transformation of ATP to ADP (8) The Na^+-K^+-dependent ATP-ase activity was calcu-

lated as the difference between the total (obtained in presence of 2 mM Mg^{2+} 80 mM Na^{+} and 33 mM K^{+}) and Mg^{2+}-dependent (obtained in presence of 2 mM Mg^{2+}) alone and expressed in micromoles of P_i/ mg membrane protein/hour The protein content was assayed by biuret reaction (20) The effects of different drugs were measured on Mg^{2+}-dependent and Na^{+}-K^{+}-dependent ATP-ase activity The drug - Na^{+}-K^{+}-dependent ATP-ase interaction was analyzed according to Ariens Simonis and van Rossum (21)

The following drugs were used for the observations:acetylcholine(Acetylcholine Chloride Fluka) atropine(Atropinum Sulfuricum Biogal Debrecen) isopropamide (2 2-diphenyl-4-diisopropylamino-methyliodide Chinoin Budapest) Gastrixon^{R}(methyltropinium-bromide-xanthene-9-carboxylate United Drug and Foodstuff Factory Budapest) ouabain (British Drug House)

The results are given as means ± SEM The correlation analysis was carried out according to Fisher (22)

RESULTS

A positive and mathematically significant correlations has been found to be present between: 1 BAO values - mucosa ATP; 2 BAO values - mucosa ADP; 3 mucosa ATP - mucosa ADP; 4 mucosa ATP - mucosa Na^{+}-K^{+}-dependent ATP-ase activity; 5 mucosa Na -K^{+}-dependent ATP-ase activity - BAO values (Table I)

The Fig 1 indicates the typical behaviours of membrane fractions from the human corpus mucosa This type of membrane ATP-ase was used for further pharmacological examinations (Figs 2 and 3)

In observations with parasympatholytics the intrinsic activity of atropine was taken to be equal to 1 0 and the effects of other parasympatholytics were compared with atropine effect (Fig 3 and Table II) Both pD_2 value and intrinsic activity of atropine is higher than those of isopropamide and Gastrixon^{R}

A positive and mathematically significant correlations has been found to be present between: a total chloride

Table I Correlations between the gastric mucosa constituents of patients with different gastric basal acid outputs (BAO)

Calculated correlation between	Regression line	r	n	p value
BAO (2 40 ± 0 34) (x)[+] mucosa ATP (472 ± 42) (y)[++]	Y = 278 x − 190	0 53	28	< 0 01
BAO (2 20 ± 0 37) (x)[+] mucosa ADP (270 ± 19) (y)[++]	Y = 139 x − 38	0 59	28	< 0 001
mucosa ATP (426 ± 98) (x)[++] mucosa ADP (245 ± 50) (y)[++]	Y = 0 48 x + 50	0 99	31	< 0 001
mucosa ATP (608 ± 182) (x)[++] Na^+-K^+-dependent ATP-ase (2 60 ± 0 38) (y)[+++]	Y = 0 001 x + 1 94	0 55	32	< 0 01
Na^+-K^+-dependent ATP-ase (3 78 ± 0 28) (x)[+++] BAO (7 50 ± 2 10) (y)[+]	Y = 0 39 x + 0 49	0 88	45	< 0 001

\+ mEq / h (means ± SEM) ++ = nmoles / mg DNA value (means ± SEM)
+++ = P_i (micromoles / mg membrane protein / hour) (means ± SEM)

Fig 1 Typical behaviour of membrane fractions from human gastric mucosa The results are presented as means ± SEM of liberated inorganic phosphorus (P_i) (in micromoles / mg memberane protein / hour) n indicates the number of patients

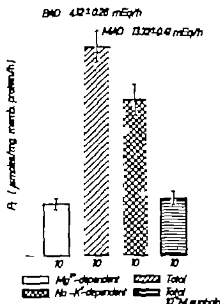
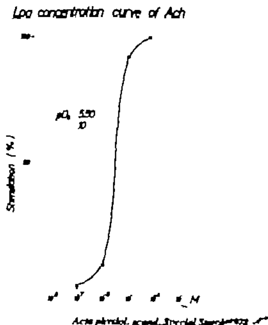


Fig 2 Log concentration dose-response curve for acetylcholine on the Na^+-K^+-dependent ATP-ase from the human gastric mucosa



Cumulative dose-response curves for parasympatholytics

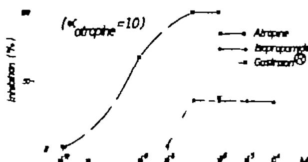


Fig 3 Cumulative dose-response curves for parasympatholytics inhibiting the Na^+-K^+-dependent ATP-ase from the human gastric mucosa The intrinsic activity (α) of atropine is taken to be equal to 1 0 on the Na^+-K^+-dependent ATP-ase activity Each point represents the average of ten measurements (isopropamide $_R$ 2 2-diphenyl-4- diisopropylamino-methyliodide; Gastrixon^R methyl-tropinium-bromide-xanthene-9- carboxylate)

Table II Values of affinities (pD_2) and intrinsic activities (α) for parasympatholytics inhibiting the Na^+-K^+-dependent ATP-ase activity from the human gastric mucosa The intrinsic activity of atropine was taken to be equal to 1 0

Parasympatholytics	Affinity (pD_2)	Intrinsic activity (α)
Atropine	8 60	1 00
Isopropamide	7 60	0.36
Gastrixon^R	6 40	0 43

(103 46 _0 36 mEq/l) (means ± SEM) - H (30 15 _ 3 50) (r = 0 466; n=32; p<0 01; Y=0 45 x + 16 62); b Ca^{2+} (4 18 ±0 82) - H^+ (29 67 ± 3 64) (r=0 496; n=31; p<0 01; Y=2 83 x + 18 00); c total chloride (103 46 ± 3 60) - Na^+ (53 60 ± 2 40) (r=0 405; n=32; p<0 05; Y=0 27 x + 25 69); d neutral chloride (74 56 ± 3 78) - Na^+ (53 60 ± 2 40) (r= 0 794; n=32; p< 0 001 ; Y=0 60 x + 16 06) ; meanwhile a negative and mathematically significant correlation was found to be present between: a Na^+ (53 60 ± 2 40) - H (30 15 _ 3 50) (r=-0 41; n=32; p < 0 02; Y=62 42 -0 60 x) and b neutral chloride (74 56 ± 3 78) - H^+ (30 16 ± 3 50)(r=- 0 501; n=32; p < 0 01; Y=84 70 - 0 46 x) in the gastric juice

DISCUSSION

Suggesting some correlation between the membrane (Na^+-K^+-dependent) ATP-ase system and the gastric H^+ secretion we must obtain reliable correlations between the enzyme activity quantity of enzyme substrate (ATP) product of enzym activity(ADP) in the human corpus mucosa and the gastric H secretion Different requirements need to prove the causal correlation between them in humans:

A mathematically significant and positive correlation needs to be present between:a BAO values and tissue content of mucosa ATP; b BAO values and tissue content of mucosa ADP; c tissue contents of mucosa ATP and ADP; d tissue contents of mucosa ATP and mucosa Na -K^+-dependent ATP-ase activity; e mucosa Na^+ K -dependent ATP-ase activity and BAO values;

B the drugs effecting on the human gastric basal acid output can modify the Na -K -dependent ATP-ase activity i n v i t r o circumstances (in the same direction as the drug effect on BAO)

These correlations have been proven in the present results (Aa;Ab;Ac;Ad) and in a previously published paper (12) These results indicate the following:

1 the increase of gastric BAO values is proportional one with the rise of corpus mucosa ATP;

2 the rise of BAO values runs paralelly with the increase of transformation of ATP to ADP by the way of Na^+-K^+-dependent ATP-ase;

3 the higher tissue content of human gastric mucosa ATP associates with a higher transformation of ATP to ADP by the Na^+-K^+-dependent ATP-ase;

4 the ratio of ATP/ADP remains about the same in the human corpus mucosa againts the different extent of ATP transformation to ADP;

5 the results were calculated in accordance to 1 0 mg DNA value which represents the same number of cells in the corpus mucosa It has been concluded from that: the biochemical composition of cells in the human corpus mucosa is different in patients with hypacidity normacidity and hyperacidity This conclusion can give two more informations:a the gastric basal acid output depends- besides the number of parietal cells - on the biochemical activity of cells too; b the results of biochemical examinations frequently differ in animals and in patients

From the study of drug effects on Na^+-K^+-dependent ATP-ase from the human corpus mucosa the following have been concluded;

1 the transformation of ATP to ADP by the way of membrane ATP-ase is open to acetylcholine effect; while it can be inhibited by parasympatholytics;

2 the pD_2 values and intrinsic activities for the parasympatholytics inhibiting the Na^+-K^+-dependent ATP-ase activity from human corpus mucosa are very different

The human gastric basal acid secretion depends on the cholinergic influences (no effect of beta adrenergic blocking agent was obtained on BAO) Probably the cholinergic effect on the human gastric secretory function depends on the number of receptors in both directions of stimulation and inhibition The observations with large dose atropine (180 ± 87 ng given intravenously; n=13) gave a good proof for this suggestion (23)

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Identity o ... n

The question resolves itself into defining whether histamine has the same action as that of other measures that stimulate gastric acid secretion (gastrin vagal stimulation distension) Or more succintly can histamine mimic the actions of gastrin or cholinergic agents? Although qualitatively there is little doubt quantitative differences exist One feature that may be fairly general is that in molar terms pentagastrin (gastrin) and in some instances cholinergic agents are far more potent than histamine in stimulating acid secretion The pentagastrin/histamine potency ratios are in the order of 100 for frog (4) 150 for rat (5) 100 for mouse (6) 500 for kitten (7) and 150 for cat dog and man (8) But reduced potency does not exclude histamine as a mediator since it is difficult to quantitate the amount of histamine actually released at the receptor site and which in fact may be several orders of magnitude greater Differences in the slopes of the dose response curves to histamine and penta gastrin have been noted in rat (5) and mouse stomachs (6) James (5) felt that this difference in the slopes indicated different mechanisms of stimulation In both instances the response curve to histamine was steeper If pentagastrin acted on the parietal cell indirectly by releasing

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Histamine as the Final Common Mediator The View From the Fence

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ABSTRACT: Two essential criteria for identifying neurotransmitters are those of the identity of action and collectibility; and the same can be applied to resolve the controversial view that histamine is the final common local chemostimulator of the parietal cell Whether in fact such criteria have been applied and to what effect are the themes of this paper

Amongst the more enduring controversies in the field of gastric physiology the concept of histamine as the final common mediator deserves honourable mention In 1938 McIntosh (1) argued that an attractive hypothesis which seems legitimate is that histamine is liberated within or near to the parietal cells as a result of stimulation of the vagus which nerve supplies these cells with secretory fibres and that the histamine thus liberated is responsible for the secretory activity of the cells Code (2) later stated that stimulation of gastric secretion is a function of histamine No other chemostimulator is interposed between histamine and the parietal cell Histamine is the final common local chemostimulator of the parietal cells of the gastric mucosa

Granted that the above proposition is true what kind of evidence would be necessary to confirm or reject it The situation is somewhat analogous to the identification of a neurotransmitter at central or peripheral synapses

Although a number of criteria have been suggested Werman (3) has noted that it is an obvious but ignored fact that only two criteria need be established in order to identify a transmitter: the criteria of identity of action and that of collectibility

The first criterion states that the actions of the putative transmitter must mimic faithfully those produced by the natural stimulus The second states that stimulation must be accompanied by the release of the transmitter in the vicinity of the synapse In the sections that follow the evidence gathered to satisfy the above two criteria will be reviewed

Identity of Action

The question resolves itself into defining whether histamine has the same action as that of other measures that stimulate gastric acid secretion (gastrin vagal stimulation distension) Or more succintly can histamine mimic the actions of gastrin or cholinergic agents? Although qualitatively there is little doubt, quantitative differences exist One feature that may be fairly general is that in molar terms pentagastrin (gastrin) and in some instances cholinergic agents are far more potent than histamine in stimulating acid secretion The pentagastrin/histamine potency ratios are in the order of 100 for frog (4) 150 for rat (5) 100 for mouse (6) 500 for kitten (7) and 150 for cat dog and man (8) But reduced potency does not exclude histamine as a mediator since it is difficult to quantitate the amount of histamine actually released at the receptor site and which in fact may be several orders of magnitude greater Differences in the slopes of the dose response curves to histamine and pentagastrin have been noted in rat (5) and mouse stomachs (6) James (5) felt that this difference in the slopes indicated different mechanisms of stimulation In both instances the response curve to histamine was steeper If pentagastrin acted on the parietal cell indirectly by releasing

histamine from a limited pool the slope of the dose response being a composite of two slopes would be different from that observed with histamine

A more serious objection relates to the maximal responses obtained with different stimulants The stimulation of acid secretion by secretagogues can be regarded as analogous to enzyme substrate reactions which can be characterised by defined affinity constants and maximal velocities If the maximal responses to different agonists are widely different it is difficult to imagine a common mechanism or a common agonist (9) The published data is however confusing In rat with gastric fistulae (10) gastrin extracts were not only more potent but the maximal responses were greater In man and cat the maximal responses were equal; in dogs with Pavlov pouches the responses were equal but in those with Heidenhain pouches the maximal gastrin response was less (11) Rosato et al (12) observed that in 3 out of 8 conscious monkeys histamine produced higher maximal responses than pentagastrin and the responses to histamine were less variable Such studies underscore the difficulties in applying sophisticated analyses (Michaelis-Menten kinetics) to systems plagued with innumerable interacting variables

Isolated tissues on the other hand have certain inherent advantages since complicating factors such as variable blood flows nervous control or diffusion barriers can be either minimised or abolished Kasbekar (4) showed that the isolated bull-frog gastric mucosa became refractory to repeated stimulation with pentagastrin or acetylcholine but that such refractory mucosae still responded to histamine This could imply that pentagastrin and acetylcholine act on the parietal cell through histamine and that exhaustion of histamine stores leads to tachyphylaxis In other isolated preparations e g isolated rabbit gastric glands (13) or the isolated piglet gastric mucosa (14) the responses to pentagastrin are either feeble or nonexistent Michelangeli

(15) reported histamine and acetylcholine stimulation of a preparation of isolated oxyntic cells from the bullfrog; the preparation did not respond to pentagastrin Isolated rat parietal cells however respond to histamine and synthetic human gastrin (16 17) Soll (18) showed that the oxygen uptake of a crude preparation of canine mucosal cells was increased by pentagastrin and urecholine but not by histamine; however morphological changes suggestive of secretion was produced by all three agonists (19)

The isolated monkey stomach stripped off its muscle coats responds to exogenous histamine in a dose-dependent fashion (Fig 1) The rates corrected for a chamber area of 3 14 cm^2 are comparable to those observed with <u>in vitro</u> frog stomachs The tissue also responds to pentagastrin; in 5 experiments the rates increased from zero to 9 2±1 7 ueq h^{-1} with 10^{-6}M agonist However so far it has been difficult to obtain clear dose-response curves with pentagastrin Occassional stomachs that did not respond to histamine did so after the addition of aminophylline (10^{-4} to 10^{-6}M) which also potentiated in general the response to histamine The preparation remains viable for 5 to 8 hours and appears suitable for studying secretagogue interactions

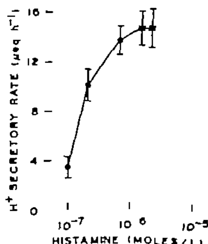


Fig 1 Cumulative dose response curve to histamine, isolated monkey gastric mucosa Mean ± SEM from 4 animals shown Spontaneous secretions nil in each case

Collectibility

Many attempts have been made to establish the second criterion that of collectibility If gastric stimulants release histamine from the gastric mucosa it is possible that this could be reflected in a reduced concentration of histamine in the gastric mucosa or an increased concentration in the gastric venous blood or in the gastric juice With isolated preparations corresponding changes would occur in the serosal or luminal bathing fluids Changes in total histamine content are likely to be least informative since small changes in large basal levels would be easily missed Furthermore there could be histamine stores that are unrelated to acid secretion Changes in the concentration of histamine in the gastric juice or luminal solutions necessitate the movement of histamine from the serosal to the mucosal side either through or between cells and here again the probability of demonstrating an increased leak seems low An increased leak from the serosal side into the serosal bathing fluids or gastric venous blood seems more likely provided that histamine being released from sites other than the parietal cell acts on receptors located on the serosal surface of the parietal cell

The methods generally used to show release of histamine have been either bioassay or fluorometry In some instances the efflux of ^{3}H or ^{14}C-labelled histamine has been studied (20 21) In all cases where tissue or plasma levels are studied separation of histamine from interfering substances becomes essential to increase sensitivity and specificity

Bioassay methods usually rely on the sensitivity of the guinea-pig ileum to histamine in concentrations as low as 5 ng/ml The contractions can be specifically blocked by mepyramine (22) The procedures though simple sensitive and specific are tedious Fluorometric methods are based on the observation (23) that histamine forms a fluorophore with O-phthalaldehyde (OPT); substances such as spermidine interfere and have to be eliminated (24) Refinements

have been made (25 26) that increase the sensitivity and the specificity of the method Both Hakanson et al (25) and Lorenz et al (26) have proposed tests of identity for histamine the most specific being degradation of the material by either purified histamine methyltransferase or diamine oxidase To date the fluorometric procedure when carried out carefully seems eminently suitable for measuring histamine release Isotopic methods for estimating histamine in plasma have been developed (27 28) but do not appear to have been widely used by gastric physiologists though the methods are sensitive enough to detect 0 3 ng/ml of histamine in plasma

Some attempts to measure changes in the histamine content of the gastric mucosa or determine histamine spill-over into the serosal (gastric venous blood) or luminal (gastric juice) fluids have been tabulated (Table 1) Various methods of stimulating gastric secretion were used by the authors cited These included for e g injection of gastrin vagal stimulation administration of carbachol etc (the original papers should be consulted for details) Both positive and negative reports have been included

TABLE 1: CHANGES IN HISTAMINE LEVELS

Samples taken from	Positive reports		Negative reports	
	Animal	Ref	Animal	Ref
Serosal side	Frog	20 29 30	Dog	37 38
	Man	31	Cat	39
Tissue	Frog	32	Frog	40
	Rat	33 34 35 36	Man	37
Mucosal side	Rat	21 33	Dog	41
	Man	37	Cat	39
			Man	31

The table though far from complete emphasises the paucity of evidence in favour of histamine as the final mediator Although some difficulties could be attributed to technical problems it is difficult to escape the conclusion that direct evidence is sound only for the frog and perhaps the rat In the case of the frog not only do gastric stimulants increase the efflux of histamine but the chemical leaking into the serosal fluid is capable of stimulating acid secretion analogous to Loewi s original experiment with acetylcholine (29) The results even in the rat are sometimes controversial Anrea and Thompson (36) noted a decrease in mucosal histamine content only in sham operated rats not in Shay rats In humans the report by Caridis et al (31) suffers from inadequate controls and also faulty statistics Rapid inactivation of histamine may pose assay problems but even the use of an inhibitor of diamine oxidase (aminoguanidine) did not significantly change histamine concentrations in gastric venous blood (37) If as pointed out by Lake (42) N-methyl histamines are the active forms measurement of histamine per se may not be revealing

But does the lack of direct evidence constitute grounds strong enough to reject the hypothesis of histamine as the final common mediator? If so is it worthwhile to continue to gather evidence? The answer to the first question is a guarded no and to the second a qualified yes There is a con iderable body of indirect evidence strongly suggesting the involvement of histamine as a link Many of the recent studies have been based on the use of H-2 antagonists (see discussion section; Bunce et al (43)) which have radically transformed this field and even Grossman (44) one of the staun chest critics of the histamine mediator hypothesis states at one point the only plausible explanation now available for the action of H-2 antagonists against many different stimulants of acid secretion is that histamine is involved in the action of other timulants But he

continues on to state his preference for a receptor interaction hypothesis

Having studied the phylogenetic distribution of histamine Lorenz et al (45) point out that there appears to be a selective accumulation of the amine in the gastric mucosae of chordates possessing acid-secreting cells and argue that this implies a specific function for stored histamine The precise function could however be different in different species The case for histamine as the final mediator is strongest in the frog but as evolution progressed receptors for other hormones may have appeared on the parietal cell histamine continuing to play a permissive role The evidence for such a view is scant at present Perhaps a broad phylogenetic study designed to satisfy the twin criteria of identity of action and collectibility may provide some answers And then again it may not - Nature has appeared to play several Enigma variations on the theme of histamine and in this instance we may well be watching the start of a somewhat elaborate fugue

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Proc. Symp Gastric Ion Transport, Uppsala 1977

Stereospecificity of d Tetrahydrozoline at H_2-Histaminergic and Alpha adrenergic Receptors

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ABSTRACT Two pharmacological actions of racemic tetrahydro zoline which are mediated by H_2 receptors its gastric secretagogue activity in isolated guinea pig stomach and its positive chronotropic effect in spontaneously beating guinea pig atria were due to the d isomer alone The alpha adrenergic agonist activity of the drug as evidenced by its vasopressor action in anesthetized cats was also produced stereoselectively by d tetrahydrozoline

Tetrahydrozoline (Figure 1) is a powerful pressor agent belonging to the imidazoline class of alpha adrenergic recep tor stimulants (1 2) In common with other closely related sympathomimetic amines such as naphazoline and oxymetazoline tetrahydrozoline is widely used as a nasal decongestant Recently Patil and co-workers (3) discovered that tetrahydro

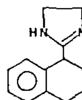


Figure 1 Tetrahydrozoline
DL-2 (1 2 3 4 tetrahydro-1 naphthyl)imidazoline

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RESULTS

The data in Figure 2 demonstrate that the positive chronotropic action of racemic tetrahydrozoline in spontaneously beating right atria from guinea pigs is due entirely to the d isomer Both isomers depress sinus rate at low concentrations an effect which is reversed by increasing concentrations of d tetrahydrozoline only

Similarly it was shown using the isolated guinea pig stomach that the gastric secretagogue activity of tetrahydrozoline is due mainly if not entirely to the d isomer as may be seen in Figure 3 The l isomer appears to be a weak antagonist of its mirror image analog with a pA_2 value estimated at 4 8 based on a single dose ratio (Figure 4)

Thus we concluded that the agonist action of dl tetrahydrozoline on atrial and gastric H_2 receptors is a property

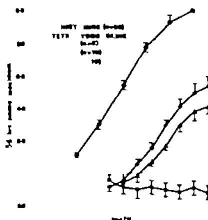


Figure 2 Positive chronotropic action of d tetrahydrozoline in spontaneously beating guinea pig atria in vitro Data ± S E

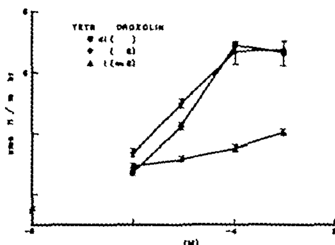


Figure 3 Secretagogue activity of d tetrahydrozoline in isolated guinea pig stomach Basal secretion (± S E) was (dl) 2 0 ± 0 2; (d) 2 3 ± 0 2; (l) 2 7 ± 0 1

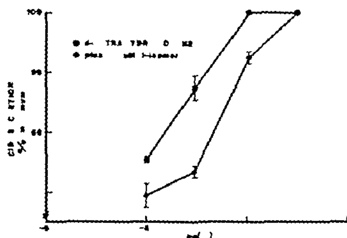


Figure 4 Inhibition of the gastric stimulant action of d tetrahydrozoline *in vitro* by the l isomer Six pieces of guinea pig stomach were used three control and three experi mental assigned at random

of the d antipode And in hopes that opposite stereospeci ficity would be found at alpha adrenergic receptors we test ed the optical isomers of tetrahydrozoline on cat blood pres sure But alas the d isomer possessed virtually all of the drug s vasopressor activity as shown in Figure 5

DISCUSSION

The chirality of tetrahydrozoline has enabled us to demon strate the stereospecific activation of H_2 receptors by the d enantiomer The tool at hand may prove useful in future investigations of histamine receptors as in the development of ligand binding assays for example This is also the first published observation that the sympathomimetic activity of tetrahydrozoline is due to its d form It may be a coinci dence that the same antipode of tetrahydrozoline activates alpha adrenergic and H_2 histaminergic receptors stereoselec tively yet this fact flashes to mind the intriguing notion

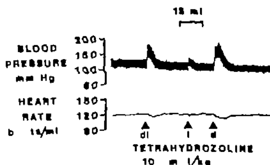


Figure 5 Typical example of the stereospecific effect of d tetrahydrozoline on femoral blood pressure and heart rate in the anesthetized cat (Chlorolose 80 mg/kg i v)

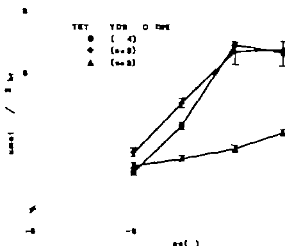


Figure 3 Secretagogue activity of d tetrahydrozoline in isolated guinea pig stomach Basal secretion (± S E) was: (dl) 2 0 ± 0 2 (d) 2 3 ± 0 2 (l) 2 7 ± 0 1

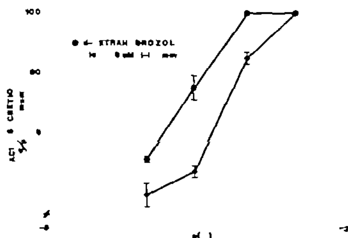


Figure 4 Inhibition of the gastric stimulant action of d tetrahydrozoline *in vitro* by the l-isomer Six pieces of guinea pig stomach were used three control and three experimental assigned at random

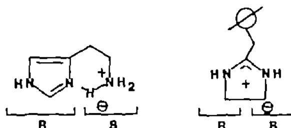


Figure 6 Hypothetical binding of histamine (left) and tolazoline (2 benzyl 2 imidazoline) to the H_2 receptor R and S refer to the ring and side chain amino binding sites of histamine respectively

we know the absolute configuration of the optical isomers of tetrahydrozoline

Acknowledgment We thank Mr Nathan Belcher of Pfizer for the stereoisomers of tetrahydrozolines

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Interactions between Intermediary Metabolism and Gastric H^+ Ion Secretion

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ABSTRACT The effects of gastric secretagogues on pyruvate oxidation were investigat d in toad gastric mucosa Theophylline but not histamine increased the rate of oxidation of labeled pyruvate; 60% of this increase was accounted for stimulation of the pyruvate dehydrogenase (PDH) complex Tracer studies showed that pyruvate carboxilation was not significantly affected by histamine and theophylline PDH activity of a mitochondrial extract was inhibited by ATP and stimulated by 20mM Mg^{2+}, indicating that the gastric enzyme is subjected to regulation by phosphorylation and dephosphorylation Theophylline 10mM did not significantly change the tissue levels of ATP ADP and the ATP/ADP ratio suggesting that the stimulatory effect of theophylline on PDH was not due to a decrease of ATP concentration 4-Pentenoic acid sharply reduced the rate of oxidation of labeled octanoate The respiratory and secretory responses to butyrate theophylline and histamine were significantly inhibited by pretreatment with 4-pentenoic acid This inhibition was partially overcome by addition of 10mM pyruvate The present results permit to suggest that activations of Beta-oxidation and pyruvate oxidation are involved in the mechanism of action of theophylline

Recent experim ntal evidence strongly suggests that the t on of secretagogues such as theophylline and histamine is mediated by intracellular 3 5 -cyclic adenosine monophosphate (cAMP) in amphibian gastric mucosa (1 4) The exact mechanism by which cAMP stimulates acid secretion is not known but current evidence indicates that the activation of oxidative metabolism plays an important role (5-8)

The observations of Alonso and col (9) that some exogenous fatty acids mimic the actions of secretagogues and the recent studies of High and Hersev (5) have led to the hypothesis that the Beta-oxidation is primarily involved in the mechanism of action of gastric secretagogues On the other hand Harris and col (8) have shown that acid secretion and oxygen uptake by frog gastric mucosa in vitro are sharply stimulated by lipoic acid Chacín and col (10) have also found that the pyruvate dehydrogenase complex of frog gastric mucosa is significantly activated by incubation with lipoate Thus the stimulations of acid secretion and oxygen uptake produced by lipoic acid have been ascribed to the increase of pyruvate oxidation due to activation of the complex These findings have led us to the question whether pyruvate oxidation is affected by gastric secretagogues or not And the present study was undertaken in an effort to answer this question In addition some inhibitory studies in relation to the role of Beta-oxidation are also presented

METHODS

All experiments were performed in vitro on gastric mucosa from fasted Venezuelan toads (Bufo marinus) Oxygen uptake (qO_2) and hydrogen ion secretion (qH^+) were measured as previously described (8 10 11)

Production of $^{14}CO_2$ from labeled pyruvate and octanoate was measured as follows preweighed paired slices of tissue were incubated 2 hours at 30 C with 2 ml of nutrient solution pH 7 4 containing 0 2 µCi of the labeled compound

Pyruvate was used at a final concentration of 10mM and octanoate at 1mM The incubation flasks were sealed after gassing with 100% O_2 and the evolved $^{14}CO_2$ was trapped in 0 2 ml of Hyamine in a small glass vial placed in the center well of the flask The incubation was terminated by an injection of 1 ml 2M H_2SO_4 and the flask was shaken for 90 min The small vial was then transferred to a counting vial with 10 ml Aquasol for liquid scintillation counting Counts of two control flasks without tissue which were run simultaneously were subtracted from the total counts per min (CPM) of the flask with tissue

The rate of pyruvate carboxilation was estimated in the intact tissue by measuring the total incorporation of label from $H^{14}CO_3$ into Krebs cycle intermediates as previously reported (10)

The effect of theophylline on pyruvate dehydrogenase (PDH) activity was studied in the following manner: preweighed paired sections of gastric mucosa were incubated one hour at 30 C with 5 ml of nutrient solution with and without 10mM theophylline At the end of incubation the tissue was quickly frozen in liquid nitrogen then pulverized with a stainless steel percussion mortar previously cooled in dry ice and finally homogenized with 5 volumes of 20mM potassium phosphate buffer pH 7 4 PDH activity was assayed in these homogenates by measuring the rate of formation of $^{14}CO_2$ from (1 ^{14}C) pyruvate (12) as described above Each assay was performed by duplicate The reaction mixture contained in micromoles potassium phosphate buffer pH 7 4 100 Cl_2Mg

2 NAD^+ 5; coenzyme A 0 5; thiamine pyrophosphate 0 4 L-cysteine-HCl 6 (1-^{14}C) pyruvate 4 (0 2 µCi) and 0 4 ml of enzyme in a total volume of 2 ml The flasks were incubated with shaking for 20 min at 37 C A blank without enzyme was also run simultaneously and its radioactivity was subtracted from the experimental flasks

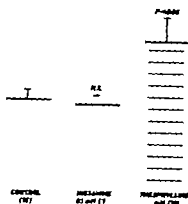
The effects of ATP and Mg^{2+} on PDH activity were also studied In these experiments the enzimatic material used was a mitochondrial extract prepared as previously reported (10) and the PDH activity was assayed by monitoring NADH formation at 340 nm with a recording spectrophotometer (10) The protein concentration was measured by the method of Lowry and col (13) with bovine serum albumin as a standard

The effects of theophylline stimulation on tissue levels of ATP and ADP were investigated in chambered mucosas Theophylline 10mM was added to one of two mucosas when the rate of spontaneous acid secretion had dropped below one $\mu Eq/cm^2/hr$ An hour later both mucosas were rapidly frozen in liquid nitrogen ATP and ADP were then extracted and assayed spectrophotometrically following the methods of Williamson and Corky (14) Hexokinase and glucose 6-phosphate dehydrogenase were used for ATP and pyruvate kinase and lactic dehydrogenase for ADP

RESULTS AND DISCUSSION

The incubation with 10mM theophylline increased the rate of $^{14}CO_2$ formation from (2 ^{14}C) pyruvate by 60% over that of control mucosas whereas 0 1mM histamine had no significant

Fig 1 Effects of histamine and theophylline on $^{14}CO_2$ - production from (2-^{14}C)pyruvate Vertical line on top of bars represents onestandard error



effect (Fig 1) These results indicate that theophylline stimulates pyruvate oxidation in toad gastric mucosa The observation that histamine did not affect pyruvate oxidation was an unexpected finding according to the common mediator (cAMP) hypothesis The exact reason for these different results is not known but there are some possible explanations Histamine is assumed to act on a membrane - adenyl cyclase (3) while theophylline on an intracellular phosphodiesterase (2) Thus one possibility is that these agents might affect different intracellular compartments of cAMP Additionally they might affect intracellular cAMP levels in different degrees

Fig 2 Outside effects of various concentrations of exogenous pyruvate on label incorporation from $H^{14}CO_3^-$ Inside reciprocal plot of velocity of incorporation and exogenous pyruvate concentration

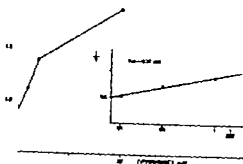


TABLE 1 Effects of theophylline ATP and a high concentration of $MgCl_2$ on PDH activity

Additions (n)	PDH activity %
None (13)	100
Theophylline 10mM (4)	133 8±4 4*
ATP 0 3mM (5)	60 9±7 7*
$MgCl_2$ 20mM (4)	149 1±6 9*

The effect of theophylline was examined as described in METHODS The effect of ATP was determined by incubating for 30 min at 30 C the following mixture 40mM potassium phosphate buffer pH 7 4 1 3mM L-cysteine 1mM $MgCl_2$ 0 3mM ATP and 0 4 ml of a mitochondrial extract in a total volume of 0 6 ml After incubation PDH activity was assayed spectrophotometrically in a 0 5 ml aliquot of the incubation mixture The effect of Mg^{2+} was tested in the same manner except that ATP was excluded from the incubation mixture and 20mM $MgCl_2$ was used instead of 1mM The control consisted of a similar mixture without ATP and $MgCl_2$ Values are means±S E
* P<0 01

The effect of theophylline on pyruvate oxidation was further examined Pyruvate is metabolized in animal tissues by two main biochemical reactions

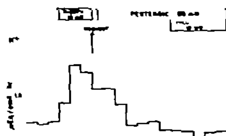


Fig 3 Illustrative experiment showing the effect of theophylline (theoph) on QH^+ in the absence and presence of 4-pentenoic acid

One is catalysed by the PDH complex which produces acetylCoA and the other one is catalyzed by the pyruvate carboxylase (PC) enzyme that produces oxaloacetate AcetylCoA and oxaloacetate are then oxidized in the Krebs cycle An increased rate of pyruvate oxidation can be due to stimulation of one or more of these pathways Table 1 shows the effects of theophylline on PDH activity Theophylline 10mM significantly increased PDH activity by 34% over the control value Therefore about 60% of the theophylline stimulation of the rate of pyruvate oxidation can be accounted for activation of the PDH complex

The rate of pyruvate carboxylation was estimated by measuring the fixation of label from $H^{14}CO_3^-$(10) This method proved to be useful to estimate the rate of the PC reaction in the intact tissue The effects of various concentrations of pyruvate on label incorporation are illustrated in Fig 2

Pyruvate increased in a dose-dependent manner the fixation of label from $H^{14}CO_3^-$ Using the exogenous concentrations of pyruvate and the Lineweaver-Burk plot an apparent

Fig 4 Inhibitory effect of 4 pentenoate on aH^+ in theophylline stimulated mucosa and lack of butyrate effect in the presence of 4-pentenoate

Km of 0 77mM was calculated (inside of Fig 2) This value is close to that of 0 4mM obtained with the isolated enzyme (10 15) The effects of theophylline and other compounds on label incorporation are shown in Table 2 Theophylline alone decreased label incorporation by 36% as compared to the untreated mucosa The rate of incorporation was similar in both pyruvate-treated and theophylline plus pyruvate-treated mucosas thus suggesting that theophylline did not significantly affect the net incorporation in the presence of an excess of pyruvate Pyruvate 10mM increased label incorporation by 58% and 161% compared with the control and theophylline-treated mucosas respectively The incorporation was not significantly changed by incubation with histamine and butyrate

The above results could be explained on the basis of a competition btaween PDH and PC for pyruvate Theophylline could favor the flow of pyruvate through the PDH complex thus reducing the availability of substrate for the PC reaction A similar mechanism has been proposed to explain the effect of lipoic acid on pyruvate carboxylation (10)

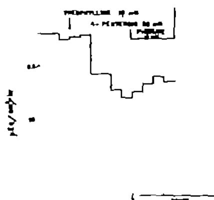


Fig 5 Effect of pyruvate on qH^+ in 4-pentenoate inhibited gastric mucosa Acid secretion was stimulated from the beginning by addition of theophylline

TABLE 2 Effects of various compounds on fixation of label from $H^{14}CO_3$ by toad gastric mucosa

Treatment (N)	$H^{14}CO_3$ fixation (µmoles/g w w)	P
Control (28)	0 89±0 06	
Pyruvate (8) 10mM	1 41±0 21	<0 025
Butyrate (6) 10mM	0 94±0 01	N S
Theophylline (14) 10mM	0 57±0 06	<0 001
Theophylline plus Pyruvate (9)	1 49±0 18	<0 005
Histamine (9) 0 1mM	0 93±0 11	N S

Values are means ± S E   N S (Not significant)

The finding that the stimulatory effect of pyruvate on label incorporation was enhanced in the theophylline-treated mucosa might be due to an increase of acetylCoA concentration which in turn might activate the PC enzyme AcetylCoA is a well known positive modulator of PC in animal tissues (10)

In an effort to determine the mechanism by which theophylline stimulated the PDH system the role of ATP was investigated It has been shown that the PDH complex can be regulated by phosphorylation (inactivation) and dephosphorylation (activation) via a kinase and a phosphatase respectively (16 17) The PDH phosphatase is activated by high concentrations of Mg^{2+} and the kinase requires ATP and low concentrations of Mg^{+} Durbin and Michelangeli (18)

have also shown in frogs that the ATP/ADP ratio is signifi cantly reduced in the histamine and theophylline-stimulated gastric mucosa Therefore the theophylline stimulation of PDH might be due to a reduction of ATP concentration

Table 1 shows the effects of ATP and 20mM $MgCl_2$ on PDH - activity of a mitochondrial extract from toad gastric mucosa.

Incubation with 0 3mM ATP significantly reduced PDH ac - tivity by 39% compared with aliquots incubated without ATP

In contrast 20mM $MgCl_2$ increased PDH activity by 49% - above the control These results appear to indicate that the PDH complex of toad gastric mucosa is also subjected to reg ulation by phosphorylation and dephosphorylation

Table 3 shows the effects of theophylline on qH^+ and the tissue levels of ATP and ADP It can be observed that theo- phylline did not significantly change the tissue levels of ATP ADP and the ATP/ADP ratio According to these results the activation of PDH by theophylline can not be explained on the basis of a reduction of ATP concentration

The effect might be directly mediated by cAMP Activation of PDH by cAMP has been reported by some authors (12 19)

However further investigation is needed to determine the exact mechanism by which theophylline stimulates PDH ac tivity in toad gastric mucosa

High and Hersey (5) have suggested that theophylline - acts by mobilizing endogenous fatty acids and that the Beta- oxidation plays a specific and direct role in the secretion of acid by frog gastric mucosa

TABLE 3 Effects of 10mM theophylline on qH^+ and tissue levels of ATP and ADP

Condition (N)	qH^+ ($\mu Eq/cm^2/h$)	ATP (nmoles/g w w)	ADP	ATP/ADP
Control (16)	0 80 ±0 14	569 9 ±89 2	254 2 ±36 9	2 13 ±0 43
Theophylline (16)	2 60 ± 0 16*	484 8 ±116 4**	280 0 ±31 8**	1 75 ±0 41**

Values are means ± S E P <0 001 ** Not significant

In the present study theophylline stimulated pyruvate oxidation and PDH activity In order to evaluate the relative contributions of pyruvate oxidation and Beta-oxidation in the mechanism of theophylline stimulation of acid secretion some inhibitory studies were conducted with 4-pentenoic acid an inhibitor whose major effect is on Beta-oxidation (20 21)

4 Pentenoic acid 10mM produced a drastic inhibition (62%) on the rate of $^{14}CO_2$ production from (1 ^{14}C)octanoate, whereas 20mM 4 pentenoic acid produced only a small reduction (22%) on the rate of $^{14}CO_2$ formation from (2-^{14}C) pyruvate and this change was not statiscally significant (Table 4) These results seem to suggest that the major inhibitory effect of 4 pentenoic acid in toad gastric mucosa is on fatty acid oxidation

The effects of 4-pentenoate on qO_2 are shown in Table 5 In the 4-pentenoate treated mucosas the qO_2 was significantly lower than in the control mucosas Pretreatment with 20mM 4 pentenoate sharply reduced the qO_2 responses to theophyl-

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The effect might be directly mediated by cAMP Activation of PDH by cAMP has been reported by some authors (12 19) However further investigation is needed to determine the exact mechanism by which theophylline stimulates PDH ac tivity in toad gastric mucosa

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TABLE 5 Effect of pretreating gastric mucosae with 4 pentenoic acid on q^{O_2} response to theophylline histamine and - butyrate

Pretreatment (N)	Addition	Oxygen uptake(µL/mg/hr) Before	After	%Δ
None (20)	0	1 48±0 11	1 48±0 11	-0 33±1 7
4 Pentenoate 20mM (12)	0	0 72±0 03	0 69±0 03	-5 04±2 4
None (7)	Theophylline 10mM	1 35±0 17	2 18±0 24	63 6±4 2*
4-Pentenoate (7)	Theophylline	0 87±0 13	1 09±0 19	24 4±4 2*
None (13)	Histamine 0 1 mM	1 35±0 10	1 91±0 11	44 3±3 9*
4-Pentenoate (8)	Histamine	0 93±0 10	1 12±0 19	14 8±6 8**
None (4)	Butyrate 10 mM	1 48±0 10	1 71±0 08	15 6±2 9*
4 Pentenoate (4)	Butyrate	0 84±0 07	0 76±0 07	9 2±2 2 N S

4 Pentenoate was added at time 0 Other compounds were added at 10 min Values are means ± S E * P<0 001 ** P<0 025 N S (Not significant)

Although the above results may suggest that pyruvate oxidation may be coupled and involved in the acid secretory mechanism they stress the important role of the oxidation of fatty acids in the mechanism of theophylline stimulation of acid secretion

ACKNOWLEDGMENTS

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Intracellular pH and Gastric Acid Secretion

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ABSTRACT: Intracellular pH of isolated frog gastric mucosa was monitored using a pH indicator dye bromthymol blue with the spectrophotometric technique Both thiocyanate (SCN) and imidazole (Im) block the stimulation of measurable acid secretion but only SCN blocks the typical alkaline shift of intracellular pH This observation supports the hypothesis that Im neutralizes pre-formed H-ion rather than inhibiting secretion Inhibition f active secretion by SCN is associated with an acid shift of intracellular pH independent of the mucosal medium pH and this shift is blocked by Im The effects of SCN and Im on secretion intracellular pH and metabolic parameters are interpreted as supporting a two-step sequential model for H-ion translocation

Gastric acid production is accompanied by an equivalent production of base which appears in the serosal extracellular fluid in the form of HCO_3 (1 2) This base formation is generally accepted and presumably gives rise to the well known alkaline tide phenomenon However the mechanisms responsible for the secretion of HCO_3 are poorly understood Thus the exact form of base generated during H production has not been identified but is po tulated to be either HCO_3 or OH (3 4) In the case of the latter conversion to HCO_3 is believed to occur by a neutralization reaction with H_2CO_3 which arises from the hydration of CO_2 (3) Similarly the site of HCO_3 production has not been identified Current hypotheses suggest that HCO_3 is formed at an intracellular site and transported via an lectrically neutral mechanism perhaps in exchange for Cl into the serosal xtracellular fluid (5 6 7)

Previous studies in our laboratory established the possibility of measuring intracellular pH in the intact functioning gastri mucosa by means of pH indicator dyes and the spectrophotometric technique (8 9)

The method basically involves incorporating an appropriate indicator dye in the case of these studies bromthymol blue (BTB) into the intact amphibian gastric mucosa and monitoring the change from acid to base form *during various physiological transitions BTB was incorporated into* tissues by preincubation in the presence of the dye (5x10 $^{-5}$M) for 1 2 hrs Following the loading" period excess dye was removed by successive replacement of the bathing media over a period of 2 3 hrs This procedure serves to remove most of the extracellular dye but results in a significant amount of dye remaining in the tissue The remaining dye is located largely at an intracellular site and is presumably bound to intracellular components BTB washout curves show at least two components The faster component with a half time of about 40 minutes may represent exchangable dye from several compartments The slow component had a half time of 174 min (S E ± 11 for 9 determinations) and by extrapolation to zero time contained 62 ± 2% of the total tissue dye This efflux rate is clearly too slow to be accounted for by simple diffusion Interpretation of washout curves is always difficult but the slower component certainly indicates that a substantial proportion of dye is not freely diffusable i e it is bound Localization of the major fraction of dye remaining after the rinsing period as intracellular was accomplished by examining BTB absorption changes associated with changing the pH and/or pCO_2 of the bathing media When the pH of the serosal bathing medium is *increased by addition of HCO_3 at constant CO_2 the BTB signal shows a* rapid response ($\tau_{\frac{1}{2}}$ = 40 sec) This response is consistent with diffusional equilibrium of HCO_3 in an extracellular space of thickness 0 5 cm in agreement with values previously determined for the serosal unstirred layer (10) The rapid response is followed by a much slower response which presumably corresponds to equilibration between extracellular and intracellular fluids In contrast changes in CO_2 tension result in a much larger BTB response for an equivalent pH change Indeed a large BTB response is obtained when CO_2 tension is changed even if the HCO_3 is adjusted so as to prevent any change in medium pH Thus we can identify a BTB containing space which is sensitive to CO_2 changes in the absence of a pH change and is slow to equilibrate with HCO_3 changes It seems unlikely that such a space could correspond to anything other than an intracellular space and we conclude that at least a portion of the BTB is located intracellularly Quantitatively the amount of

intracellular dye can be determined by measuring the total dye content and correcting for extracellular dye Measurements of this type have shown that less than 10% of the dye content is accounted for by extra cellular dye after the appropriate rinsing period

The basic conclusion that can be drawn from these preliminary studies is that most f the BTB signal arises from intracellular dye and therefore we are indeed measuring intracellular pH rather than extracellular pH

Previous studies (9) indicated that there is an alkaline shift in the intracellular pH associated with the onset of acid secretion and these observations were quantitatively confirmed (11) Thus the BTB signal appears to reflect the basic separation of H and OH occurring during acid production Therefore the measurement of intracellular pH with BTB can under most conditions provide an independent index of the acid secreting status of th gastric mucosa This capability has been utiliz ed to gain further information on the mechanism of action of several agents which influence acid production Of particular interest are the apparently selective agents thiocyanate (SCN) and imidazole

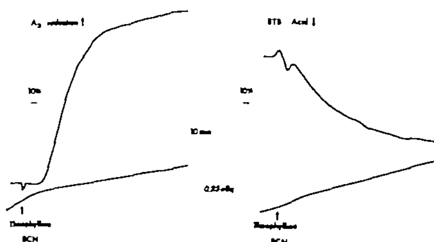


Fig 1 SCN dissociation of metobolic and secretory actions f theophyl line Simultaneous addition of theophylline (1x10 $^{-2}$M) and SCN (5x10^{-2}M) results in no increase or a slight inhibition of acid production The cytochrome a_3 ignal (left) shows typical reduction indicating increased metabolic activity but the BTB signal (right) shows an acid response typical of secretory inhibition Records were obtained on separat tissues from the same animal

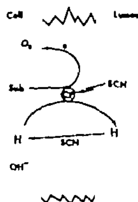


Fig 2 A model to explain SCN uncoupling action Substrate (Sub) oxidation resulting in oxygen consumption is obligatorily linked by coupling factors (CF) to H^+ translocation SCN may act directly on the coupling factors or may mediate H^+ re-entry into cell

Previous studies have shown that SCN uncouples acid secretion from oxidative metabolism (12) i e SCN inhibits H^+ secretion but does not inhibit or even increases respiration Moreover at appropriate doses SCN also permits the typical reduction of respiratory chain components associated with secretory stimulation but prevents the increase in H^+ production (13) The concept that SCN does prevent the fundamental separation of H^+ and OH is supported by the observation that the alkaline shift of intracellular pH associated with theophylline stimulation is prevented by SCN (figure 1) The apparent uncoupling of acid production and metabolism could be explained in at least two ways a true uncoupling or a shunting of H^+ These possibilities are illustrated in figure 2 In this schematic model H^+ translocation is coupled to processes which oxidize a substrate and donate elections to the terminal acceptor oxygen SCN could be envisioned as acting on hypothetical coupling factors (CF) to remove the obligatory coupling and permit the metabolic reactions to occur without H movement Alternatively SCN could act as a H^+ shuttle or protonophore to allow secreted H-ions to re-equilibrate with cytoplasmic OH In this case H^+ secretion would actually be ongoing but few H^+ would ever reach the mucosal medium to be measured by the pH electrode as actual secretion Both mechanisms would effectively prevent H^+ and OH separation and therefore prevent the usual alkaline shift of intra cellular pH while permitting metabolic events to continue At present a rigorous distinction between the two possibilities cannot be made Earlier studies provided suggestive evidence that SCN acted as a true uncoupler rather than a protonophore This evidence involved the obser vation that SCN increases tissue resistance (14) while a protonophore

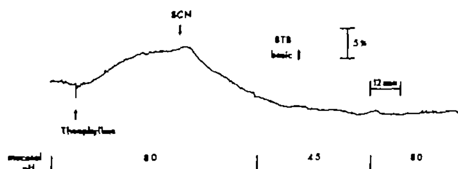


Fig 3 BTB response to SCN at pH 8 0 Resting tissue was stimulated by theophylline (1x10 ²M) with mucosal pH at 8 0 Following BTB response SCN (1x10 ²M) was added to serosal side After BTB response mucosal pH was changed to 4 5 and subsequently returned to 8 0 with no apparent change in intracellular pH

mechanism would predict the opposite In addition if SCN acts as a protonophore we would anticipate that the intracellular pH would become sensitive to changes in mucosal pH Changing the mucosal pH over a wide range (pH 4 pH 8) does not appear to influence intracellular pH as indicated by BTB in the presence or absence of SCN (figure 3) Moreover the acidification produced by SCN in association with secretory inhibition occurs just as well when the mucosal pH is 8 0 as when it is 4 5 (figure 3) These results indicate that SCN does not induce a H con ductance between the lumen and cytoplasm. The possible conclusion that SCN acts as true uncoupler however must be re-examined in view f some recent observations on the mechanism of secretory inhibition by imidazole and the interaction of this agent with SCN (15 16)

Th inhibition of acid secretion by imidazole was originally reported by Alonso Rynes and Harris (17) as a true inhibition possibly due t stimulation of cyclic nucleotide phosphodiesterase More recently Sanders t al (15 16) have postulated that imidazole does not inhibit secretion but rather neutralizes the H^+ as it is produced Thus at pH 4 5 (below pK of imidazole) H secretion is not observed but at pH 8 (above pK) secretion does occur in the presence of imidazole We have confirmed the observations of Sanders et al in that the apparent ecretory rate in the presence of imidazole is dependent upon the pH of the mucosal medium (figure 4) The pH dependence of the apparent secretion is at least qualitatively consistent with pH-dependent neutralization

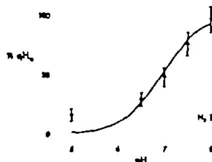


Fig 4 pH dependence of imidazole inhibition of acid production Tissues were stimulated to secrete with theophylline ($1x10^{-2}M$) and after obtaining an initial rate (qH_0) with mucosal pH at 5 0 imidazole($2x10^{-2}M$) was added to serosal medium Mucosal pH was varied randomly over range of 5 0 to 8 0 and secretion measured The values are mean ± S E for 8 determinations The point labeled N_2 represents secretion at pH 8 0 with nitrogen anoxia The solid line is a theoretical curve for imidazole dissociation

process and appears to be a true secretion as evidenced by anoxia inhibition Moreover addition of imidazole to tissues brought to secretory rest with metiamide does not induce "secretion even with the mucosal pH at 8 0 further showing that passive equilibration of imidazole cannot account for the observed acid production

It is possible though unlikely that imidazole actually inhibits secretion but the inhibition is dependent upon the pH of the mucosal solution To test this possibility we sampled the mucosal bathing medium during imidazole inhibition with the pH-stat set at pH s from 5 0 to 7 5 These samples were then titrated to pH 8 0 The amount of "titratable acid was added to the observed acid production and the total acid formation compared to the rate of secretion before imidazole addition Table 1 shows that the total acid was essentially 100% of the initial secretory rate at all pH settings Finally figure 5 shows that imidazole with mucosal pH 4 5 prevents apparent acid secretion in response to theophylline but the alkaline shift of intracellular pH still occurs This

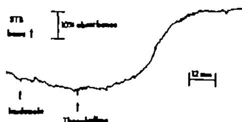
TABLE I

Total Acid Production with Imidazole

pH	Measured	Titratable Acid % Initial[a]	Total
7 5	76	33	109±8
7 0	48	53	101±10
6 5	29	62	91±10
5 0	16	87	103±5

a Measured acid is amount measured with pH stat set to pH value indicated; titratable acid is measured by titration of medium to pH 8 0 total acid equals measured plus titratable values are mean ± S E of 8 determinations Initial acid production measured at pH 5 0 with $1x10^{-2}M$ theophylline prior to addition of imidazole $2x10^{-2}M$

Fig 5 BTB response to theophylline in presence of imidazole Tissue was treated with metiamide ($1x10^{-4}M$) to reduce spontaneous secretion and then exposed to imidazole ($2x10^{-2}M$) for 25 min prior to addition of theophylline ($1x10^{-2}M$) No acid secretion was measurable during the recording period with pH-stat set at 4 5



measurement indicates that H^+ and OH are still being separated although H secretion cannot be detected by the pH-stat It should be pointed out that although an alkaline response is observed the response is attenuated by addition of imida ole to the serosal medium This attenuation is due at least in part to an intracellular buffering action of imidazole However since this buffering action is unrelated to the secretory inhibition (unpublished results) the attenuation does not alter the conclusions drawn from the experiment of figure 5

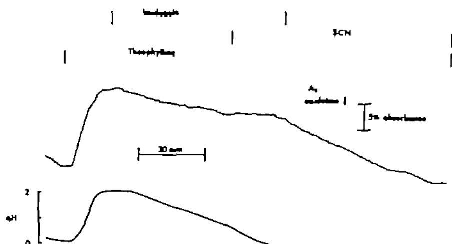


Fig 6 Cytochrome responses to theophylline imidazole and SCN Spontaneous secretion was inhibited with metiamide ($1x10^{-4}M$) and the tissue restimulated with theophylline ($1x10^{-2}M$) After cytochrome signal and secretion had reached steady level imidazole ($1x10^{-2}M$) was added to th serosal medium. Lower trace shows acid production ($\mu eq/cm^2hr$) with pH-stat set t 4 5 SCN ($1x10^{-2}M$) was added to mucosal solution At point indicated serosal solution was replaced to remove imidazole but SCN remains in mucosal s lution

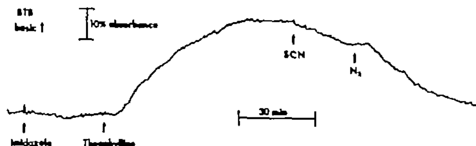


Fig 7 BTB response to SCN and anoxia in presence of imidazole Spontaneous secretion was inhibited with metiamide (1×10^{-4}M) and imidazole (1×10^{-2}M) added to serosal medium Tissue was re-stimulated with theophylline (1×10^{-2}M) but only a slight secretion (less than 0 5 µeq/cm²hr) was observed with pH-stat at 4 5 SCN (1×10^{-2}M) was added to mucosal solution and tissue was made anoxic by switching to a nitrogen gas phase

These results provide independent confirmation of the hypothesis that imidazole acts by neutralizing pre-secreted H rather than preventing H^+ and OH separation It is critical to recognize that the H imidazole interaction occurs after the H OH separation in view of the recent finding by Sanders et al (15 16) that imidazole prevents the inhibition of acid secretion by SCN This rather surprising observation is extremely important because it indicates a site of interaction between SCN and imidazole Again we have obtained independent confirmation of the imidazole block of SCN action Figure 6 shows that in the presence of imidazole SCN fails to produce its usual oxidation of cytochrome a_3 (12) Likewise the SCN acidification of intracellular pH is greatly attenuated when imidazole is present (figure 7) although anoxia produces its usual

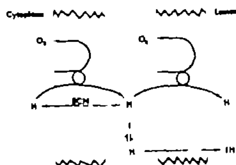


Fig 8 A two barrier model for gastric acid production

acid response Thus imidazole appears to interact with SCN so as to prevent or at least attenuate the usual actions of SCN on acid production cytochrome redox state and intracellular pH

A major assumption must be made concerning the interaction between SCN and imidazole This assumption is that the interaction occurs at the same site as the normal inhibitory actions of these agents i e the interaction occurs at the same site that SCN exerts its uncoupling effect and this site is where imidazole normally neutralizes the H The alternative is to postulate that imidazole blocks the SCN action by a mechanism which is essentially independent of its secretory neutralization mechanism A distinction between these possibilities is not possible with existing data but represents an important goal for future investigation If we accept the hypothesis of a common action interaction site the present data provide strong support for a two-step sequential mechanism for H secretion Since the imidazole neutralization occurs after the H OH separation SCN must uncouple by allowing a recombination of H and OH i e it must act as a protonophore However the increased H permeability cannot occur between the cytoplasm and the lumen since resistance and intracellular pH measurements are not compatible with such a mechanism Therefore the proton gradient which is collapsed by SCN must occur between the cytoplasm and a compartment which is normally impermeable to H either from the lumen or the cytoplasm Figure 8 illustrates one model which could account for most of the present data The system involves two barriers and an intermediate compartment which could be intramembranal or correspond to a membrane bound cytoplasmic structure The first barrier on the cytoplasmic side would perform an initial separation of H and OH and this process is assumed to be coupled in some way to oxidative metabolism as evidenced by anoxia inhibition of secretion with imidazole This barrier would also be the site of protonophore action by SCN H-ion within th intermediate compartment is normally transferred across the second barrier by a specific conductive mechanism also assumed to be coupled to metabolic processes It is within the intermediat compartment that imidazole neutralizes the H and thereby prevents SCN from acting The imidazolium ion is then capable of crossing the second barrier either by utilizing the same mechanism as the H alone or by diffusing as a permeant cation In the case of the latter some reduction in energy metabolism would be expected in accord with the

original observation (17) that imidazole inhibits respiration Addition al details and modification of this model must await a complete investi gation of various parameters influenced by SCN and imidazole however the present model seems to account for the major observations

While alternative models are possible they would deviate from the proposed one only in specific detail Within the framework of the assumed common action interaction site for SCN and imidazole any model would have to include two barriers and an intermediate compartment It is the testing of that assumption rather than the specifics of the model which would represent true progress in this area

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Microtubule Disrupting Agents and Gastric Secretion

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ABSTRACT The effects of microtubule disrupting agents colchicine and vinblastine and of microfilament disrupting agent cytochalasin B on acid and pepsinogen by in vitro preparations of bull frog gastric mucosa were studied Vinblastine (10^{-5} 5 X 10^{-4}M) added to the serosal side inhibited both H^+ and pepsinogen secretory rates in a dose-dependent manner Colchicine (1 50 mM) on the other hand transiently stimulated acid secretion over and above that obtained with supramaximal dose of histamine and then reversibly inhibited secretion Unlike vinblastine colchicine thus exerts a biphasic effect on H^+ rate In resting mucosae colchicine alone in the absence of added secretagogues initially stimulated acid secretion and this was then followed by an inhibition as in spontaneously secreting mucosae stimulated with histamine Cytochalasin B (10^{-6} 10^{-3} M) was without effect on either H^+ or pepsinogen secretion The data suggest that microtubules but not microfilaments may be involved in gastric secretion although the relatively high concentrations of colchicine required for inhibition indicate that colchicine inhibition may also be mediated by non-specific interactions with the secretory cell plasma membrane

The gastric secreting cell undergoes major ultrastructural changes when the resting stomach is stimulated to activity or the secreting tissue is brought to a resting state (1 2 3) Both mammalian as well as the amphibian cells have been shown to be characterized by such changes Recent demonstrations of the presence of K^+ stimulated ATPase in a purified microsomal fraction of gastric mucosa (4) and the association of this enzyme with vesicles involved in K^+-H^+ transport and thus possibly in H^+ secretion (5 6) suggest the following two possibilities for the ultra

original observation (17) that imidazole inhibits respiration Additional details and modification of this model must await a complete investigation of various parameters influenced by SCN and imidazole however the present model seems to account for the major observations

While alternative models are possible they would deviate from the proposed one only in specific detail Within the framework of the assumed common action interaction site for SCN and imidazole any model would have to include two barriers and an intermediate compartment It is the testing of that assumption rather than the specifics of the model which would represent true progress in this area

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side Both bathing solutions were gassed with 95% O_2 5% CO_2 gas mixture Histamine at 0 1 mM concentration was included in the nutrient bathing medium unless otherwise stated Acid secretion was monitored by the pH stat method with the endpoint setting of 4 5 Pepsinogen secretion was determined by replacing the luminal bathing solution hourly and assaying pepsin activity in an aliquot of this solution by the method of Anson and Mirsky (17) as modified by Schlamowitz and Peterson (18) Pepsin activity is expressed as pepsin units (PU) (19) Pepsinogen levels in tissues were determined by homogenizing the tissue in 5 mM acetate buffer pH 4 0 and measuring pepsin activity in an aliquot of 10 000 X g supernatant fraction of the homogenate In all studies one mucosal half was generally used as the control while the other half was incubated with an appropriate microtubule or microfilament disrupting agent added to the nutrient bathing medium

In a few instances ultrastructural studies were carried out on mucosal halves from the same epithelium. Both halves were stimulated with 10^{-4}M histamine and one half treated with colchicine while the other was used as control When the H^+ rate in the colchicine treated half declined to about 50% both halves were fixed in 2% glutaraldehyde in 50mM cacodylate buffer pH 7 4 post fixed in OsO_4 embedded in epon and thin sections at tubular levels examined by transmission electron microscopy

RESULTS

Effects of vinblastine on H^+ and pepsinogen secretion

The effect of 0 5 mM vinblastine on H^+ rate and decline of mucosal pepsinogen content is shown in Figures 1 and 2 respectively The effective inhibitory concentrations ranged from 10^{-5} to 5 x 10^{-4} M and the H^+ rate inhibition almost paralleled that of pepsinogen as ascertained from relatively constant PU/H^+ ratios (approx between 6 8)

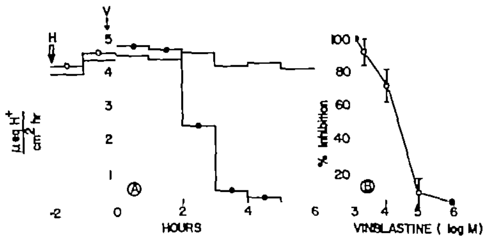


Fig 1 Vinblastine effect on H^+ secretion in histamine stimulated mucosal halves
A H^+ rate — histamine control (histamine added to both halves at H)
-• vinblastine (5×10^{-4} M added at V) treated half
B Concentration dependence of H^+ rate inhibition % inhibition in each experiment was determined by setting the 5th hour H^+ rate of the control half arbitrarily to 100% and subtracting the corresponding H^+ rate of the vinblastine treated half as % of the control rate

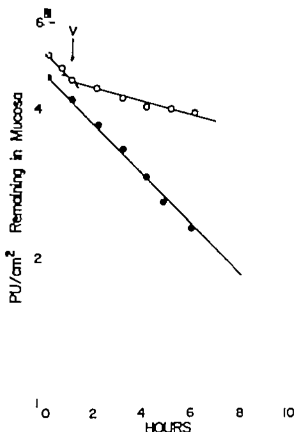


Fig 2 Pepsinogen loss from histamine stimulated mucosal halves and inhibition by vinblastine Histamine (0 1 mM) was added to both halves and vinblastine to one half at 'H and V respectively Mucosal pepsinogen levels at various times were calculated by adding the amounts of zymogen secreted at hourly intervals into the luminal bathing media to the amounts remaining in the respective mucosal halves at the end of the experiment and then subtracting the cumulative amounts secreted during successive intervals -e histamine control; o- vinblastine treated half Similar studies were carried out with colchicine and the first order rate constants for both vinblastine- and colchicine-treated mucosae were computed from the linear portions of the curves before and after addition of the agents The data are summarized in Table 1

during the first two to three hours of incubation The kinetics of inhibition were slow and at 5×10^{-4} M 5 6 hours were required to reduce H^+ secretion to zero Although H^+ rate was completely abolished between the 3rd and 4th hour at 1 0 mM vinblastine concentration pepsinogen continued to leak at a measurable rate (0 031 hr^{-1}) which perhaps represents a slow basal leak of the zymogen from the tissue

Effects of colchicine on H^+ and pepsinogen rates

Unlike vinblastine colchicine at 1 50 mM concentrations had a biphasic effect on H^+ secretion (Fig 3) There was an initial stimulation of H^+ rate (15 25%) over and above that observed with maximal histamine at all concentrations ranges of colchicine studied and this was followed by inhibition The stimulation persisted for several hours at lower concentrations However at concentrations of colchicine 25 mM and above the stimulation lasted for 1 2 hours and was followed by a rapid inhibition Renewal of the bathing medium containing fresh colchicine resulted in a partial and transient reversal of the inhibition These observations suggest that colchicine is metabolized by the mucosal cells and that a metabolic product(s) may be responsible for the biphasic effect Colchicine (10 mM) addition in the absence of exogenous secretagogues can induce secretion in the resting mucosa obtained by prolonged preincubation technique described previously (16); this stimulation of secretion was comparable to that obtained with 10^{-4} M histamine But unlike the sustained stimulation observed with histamine the initial stimulation with colchicine was followed by an inhibition similar to that described in Figure 3

The predominant effect of colchicine was however to inhibit secretion At concentrations lower than 1 0 mM colchicine had little effect on secretion although the transepithelial potential difference (PD)

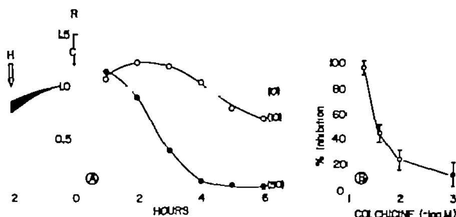


Fig 3 Effect of colchicine on H^+ rate in histamine stimulated mucosal halves histamine was added to both mucosal halves at H A Ratio (R) of instantaneous H^+ rate of the colchicine treated half (colchicine added at C) to that of the corresponding histamine control half at various times Figures in parenthesis indicate colchicine concentrations The heavy shaded line depicts the range of similar ratios for the control halves during the experimental period and was determined from instantaneous H^+ rate to the maximum rate obtained for each control mucosal half B Dose dependence of H^+ rate inhibition calculated as in Figure 1

momentarily increased (ΔPD 5 $^+$ 2 mV) and then returned to the baseline levels in about 10 min At higher concentrations the transient rise in PD was similar but the return was to lower values with a concommittant rise in resistance The kinetics of inhibition were slow and prolonged incubation at concentrations above 10 mM eventually reduced the secretion to zero with a simultaneous increase in resistance (ΔR 60 $\pm$ 10 ohm cm^2)

and decline of PD to almost zero These effects were reversible following washouts of colchicine

Pepsinogen secretion unlike H^+ secretion was not transiently stimulated by colchicine and the inhibition of pepsionogen secretion usually preceded that of H^+ secretion and was greater than that of H^+ rate Consequently it is noteworthy that with both vinblastine and colchicine the pattern of inhibition of pepsinogen secretion vis a vis H^+ secretion differs from that observed with thiocyanate a classical inhibitor of H^+ secretion or with burimamide With colchicine the PU/H^+ ratios tended to decrease from an average of 7 before addition of colchicine to about 2 at the end of 5th hour of 10 mM colchicine treatment Colchicine stimulation of H^+ rate could only partly account for this decline of PU/H^+ When vinblastine was the inhibitor the two secretions declined in an almost parallel manner so that the PU/H^+ ratios remained relatively constant until the H^+ rate approached zero late in the experiment and the continued basal leakage of pepsinogen began to increase the ratio On the other hand with 20 mM thiocyanate or 1 mM burimamide the inhibition of H^+ rate preceded that of pepsinogen and the PU/H^+ ratio increased sharply to 30 or greater at one hour after treatment and continued to increase during the next two hours The basis for differences in the action of microtubule disrupting agents versus that of thiocyanate or burimamide on these secretions is presently not clear and it is tempting to ascribe it to the possible action of the former on microtubules

Effect of cytochalasin B

Cytochalasin B had no effect on either H^+ or pepsinogen rate in the concentration range of 10^{-6} 10^{-3} M It also appeared not to modify the effects of colchicine or vinblastine in any manner

Ultrastructural studies

Typical electron micrographs of the histamine-stimulated and histamine stimulated colchicine-inhibited mucosal halves are shown in Figure 4



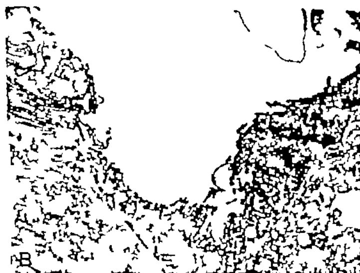


Fig 4 Ultrastructural changes in the apical regions of tubular cells in histamine treated mucosal halves and effect of colchicine
A Histamine treated control half at the end of 4th hour of incubation The H^+ rate declined from 4 4 at the start to 4 1 μeq cm $^{-2}$ hr $^{-1}$ at the end of experiment X15,000
B Colchicine treated half Colchicine (25 mM) was added after 2 hours of incubation in the continued presence of histamine The H^+ rate was 4 8 at the start 4,7 at the time of addition of colchicine and 2 8 μeq cm $^{-2}$ hr $^{-1}$ just before fixation X10 000
Apical regions in the histamine treated control mucosal half display the typical microvilli extending into the tubular lumina in almost all sections examined In contrast colchicine treatment results in resorption and stacking of the microvilli forming multilaminar structures along the apical plasma membrane

At the time of fixation the colchicine treated mucosa was secreting at approximately 60% of the control The tubular cell apical regions in the control mucosal half typically show an abundance of microvilli protruding into the tubular lumina with a depletion of cytoplasmic tubulovesicular elements In contrast in the colchicine treated mucosal half the microvilli appeared to be stacked along the plasma membrane surface forming multilaminar structures Our fixation techniques were not designed to observe microtubules or microfilaments and therefore none are visualized in these micrographs

DISCUSSION The data presented in this report clearly indicate that microtubule disrupting agents colchicine and vinblastine inhibit gastric H^+ and pepsinogen secretion whereas cytochalasin B which interacts with microfilaments is without effect on gastric secretion The requirements of high concentrations of colchicine some 2 3 orders of magnitude greater than those needed for disruption of microtubules in other secretory tissues or cells in which microtubule involvement has been reported suggests that colchicine may also react non-specifically with cellular membranes and thus exert its inhibitory effects However the concentrations of vinblastine appear to be in a reasonable range for a specific interaction with microtubules Microtubules are known to be present in

mammalian parietal cells but their functional relationship to secretion is unknown It is possible that they may play a role in the alignment of tubulovesicles prior to their fusion to the apical plasma membrane or in their eversion with the initiation of secretion and/or reversal of the above processes with a decline of secretion to the resting state While our studies thus suggest that microtubules may be involved in gastric secretion additional evidence will be clearly required before they can be definitively implicated directly in the sequence of events leading to secretion

ACKNOWLEDGMENTS

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I am grateful to Professor George B Chapman of Georgetown University for assistance with the electron microscopic studies

TABLE I

Rate constants for loss of pepsinogen content in histamine-stimulated and vinblastine or colchicine treated mucosae

Vinblastine (M)	k (hr^{1})	Colchicine (M)	k (hr^{1})
1×10^{5}	0 113	1×10^{-3}	0 117
1×10^{-4}	0 048	1×10^{-2}	0 092
5×10^{-4}	0 042	$2\ 5 \times 10^{2}$	0 046
1×10^{-3}	0 033	5×10^{2}	0 035

The rate constant for the uninhibited mucosae averaged 0 124 hr^{1}

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Cytochrome b_5 and K^+-PNPPase Specific Characterization in the Isolated Rat Gastric Parietal Cell

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ABSTRACT: A specific approach to the characterization of the gastric H^+-pump was attempted using isolated cells from rat fundus Cells were sorted by isopycnic equilibration in sucrose gradient and characterized for marker enzymes as well as for components possibly relating to H^+-transport K^+-ATPase activity was found to be lacking K^+-PNPPase and cytochrome b_5 copurified with parietal cells Cells were then subfractionated by differential centrifugation and isopycnic equilibration Both K^+-PNPPase and cytochrome b_5 shown a subcellular distribution consonant with a possible location on apical membrane and/or tubulovesicular system Digitonin treatment however allowed to separate membranes bearing K^+-PNPPase from those bearing the cytochrome on the basis of difference in cholesterol content

INTRODUCTION

The gastric H^+-pump is very likely specific of the parietal cell Morphological events accompanying parietal cell activation and deactivation have furthermore suggested its probable location on the smooth endoplasmic reticulum membranes forming the so called tubulovesicles (1 2) Prominent within the cytoplasm of the resting cell the tubulovesicles are believed to incorporate with the apical plasma membrane (the intracellular canaliculi) on the onset of stimulation (3 4) On these grounds the biochemical identification of the pump may be usefully approached in terms of isolation and purification of parietal cell membranes

Following this approach however one is faced with the embarrassing problem of the cellular heterogeneity of the gastric mucosa In the lack of more definite preparation previous attempts have focussed on microsomal fractions actually

representative of the whole tissue Availability of reliable method for gastric cells isolation and quantitative sorting (5) may now allow more specific investigations to be attempted

The present work is concerned by the biochemical study of the isolated rat parietal cell and particularly by the characterization of its smooth surfaced microsomal membranes In consideration to their possible key role a special attention was paid to microsomal ion-sensitive ATPases in particular K^+-stimulated ATPase (6-9) K^+-stimulated phosphatase (K^+-PNPPase) (10) and microsomal cytochrome b_5-linked oxido-reduction system (10 11) Also are reported first attempts dealing with discrimination between smooth endoplasmic reticulum and plasma membrane markers on the basis of the digitonin-shift model previously reported for liver (12)

MATERIAL and METHODS

Male Wistar rats weighing between 250 and 300 g were used in this study

Cell isolation and sorting

Gastric cells were prepared according to Lewin et al (5) Stomachs (fundic part) were everted mucosa outside and immersed in an oxygenated Krebs-Ringer medium at 37 C Pronase (Merck A B Darmstadt RFA; 1000 IU ml^{-1}) was injected on the serosal side and allowed to dissociate the connective stroma for 90 min Individualized mucosal cells were collected and sorted by an 1 hour equilibration in a linear sucrose gradient (PR 6000 centrifuge with CF 6 continuous flow zonal rotor IEC Needham Heights Mass USA) Cytochrome c oxidase and pepsin were used as biochemical markers to characterize parietal and peptic cells respectively Morphometric analysis of cell diameters and electron microscopic observations were performed as previously described (5)

Subcellular fractionation

Differential centrifugation was performed as previously described (13) (N) fraction 200 x g during 6 min; (M) fraction 9 500 x g during 3 min and 2 s; (L) fraction 37 000 x

g during 6 min and 42 s; (P) fraction 92 000 x g during 30 min; (S) fraction supernatant of fraction (P) In these subcellular fractions enrichment over the homogenate was estimated according to De Duve (14) in terms of Relative Specific Activity (RSA) as defined by (% of enzyme activity or biochemical constituent) : (% of protein)

Isopycnic equilibration was achieved in a 10-60% linear sucrose gradient (Spinco Beckman Centrifuge SW 25 rotor 16 h at 15 000 rpm) (13) Treatment with digitonin (Merck A B Darmstadt RFA) was performed according to Amar-Costesec et al (12) by incubating prior to isopycnic equilibration the post-mitochondrial fraction 15 min at 0 C in the presence of digitonin (3 3 mg/g of fresh mucosa) The material was then washed once by centrifugation (100 000 g x 1 h) and resuspended in 0 25 M sucrose

Biochemical determinations

All the substrates used were obtained from Sigma Chemical Co Proteins were determined by the method of Lowry et al (15) using bovine serum albumin as standard Cholesterol was assayed by the method of Rudel and Morris (16) Cytochrome c oxidase was estimated from Cooperstein and Lazarow (17) Monoamine oxidase was determined by the method of Weissbach et al (18) Pepsin was assayed by a modification of the method of Anson and Mirsky (19) as previously described (20) RNA was determined following the method of Schneider (21) Assays for 5 nucleotidase and ATPase activities were performed as previously described (13) Galactosyl transferase was assayed as described by Beaufay et al (22)

Assays for paranitrophenyl phosphatase activity were performed in a medium containing 4 mM paranitrophenyl phosphate (PNPP) 80 mM Hepes buffer (pH 6 9) with 4 mM $MgCl_2$ as an activator and with or without 20 mM KCl (23) The difference between activity in the presence and absence of potassium will be referred as to K^+-PNPPase activity

Cytochrome b_5 was determined according to the method of Klingenberg (24) from the NADH reduced minus oxidized difference spectrum using a differential wavelength scanning

spectrophotometer (Gilford Instrument Laboratories Inc Oberlin Ohio USA) Concentrations in cytochrome b_5 were calculated assuming that $\Delta\epsilon(424 - 405)$ nm = 160 $cm^{-1}mM^{-1}$ (24)

All determinations were performed under linear assay conditions with respect to protein or cell concentration in the test

RESULTS and DISCUSSION

Characterization of isolated rat parietal cells

Isopycnic equilibration of isolated fundic cells was previously detailed (5) It resulted in a clear separation between parietal cells on the one hand and peptic and mucous cells on the other: parietal cells as characterized by (a) morphologically their large size (16±4μm) the presence of intracellular canaliculi and the peculiar abundance of mitochondria (fig 1) and (b) biochemically cytochrome c oxidase as marker of mitochondria equilibrated with a mean buoyant density of 1 18-1 20 g cm^{-3}; peptic and mucous cells as identified by their smaller size (9±3μm) and their secretory granules showed a distribution paralleling that of pepsin with a mean buoyant density of 1 23-1 24 g cm^{-3} (fig 2a)

Consistently parietal cells were characterized for biochemical components possibly relating to H^+-transport with the assumption that these components would have throughout the gradient a distribution closely similar to that of cytochrome c oxidase

According to this model both cytochrome b_5 along with its associated reductase (NADH: cytochrome b_5 oxido reductase E C 1622) and K^+-PNPPase appeared to be typical of parietal cells (fig 2b) 93 5% of the whole cytochrome and 95% of the whole K^+-PNPPase were recovered in the 1 17-1 23 g cm^{-3} density band which contained 89% of the whole parietal cells The 1 23-1 30 g cm^{-3} density band which accounted for 80% of peptic and mucous cells contained only 6 5% and 5% of the whole cytochrome b_5 and K^+-PNPPase respectively In the 83% parietal cell population collected from 1 18-1 20 g cm^{-3} the cytochrome was enriched 3 1 times and K^+-PNPPase 3 3 times over the values

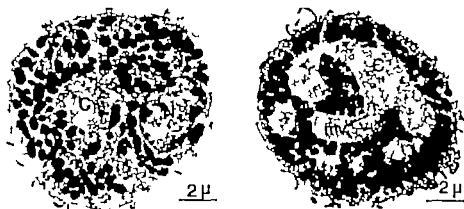


Fig 1 Electron micrographies of isolated rat parietal cells N nucleus; M mitochondria; L lysosome; TV tubulovesicles; C intra cellular canaliculi. On the left inactive cell; on the right active cell showing prominent canaliculi with profusion of microvilli (mv) These microvilli should be produced by tubulovesicles fusing with the intracellular canaliculi membrane (1 4)

prior cells separation For a pure 100% parietal cells population extrapolated contents were 19 6 pmoles and 10 6 nmoles min^{-1} for cytochrome b_5 and K^+-PNPPase respectively

The specific occurence of cytochrome b_5 in parietal cells is in apparent agreement with predictions of the redox pump hypothesis for H^+-transport (25 26) and furthermore consistent with previous evidence on rabbit gastric microsomes (10) This finding is detailed elsewhere (11) Gastric K^+-PNPPase is commonly suggested to be associated with the so called K^+-ATPase (6) This latter enzyme has been characterized in gastric microsomes from various amphibian and mammalian species including rat (27) It was claimed to be unique of the gastric tissue (7) and strongly suggested to drive H^+ transport according to the ATPase pump hypothesis (4-9 28 29) However in sharp contrasts with these reports isolated rat fundic cell were found to be devoided of any detectable K^+-stimulable ATPase activity We have no definite explanation for this striking result It might for instance reflect the occurence under our experimental conditions of either a damage of a necessary cofactor or a conformational change in the enzyme molecule resulting in unsensitivity to K^+

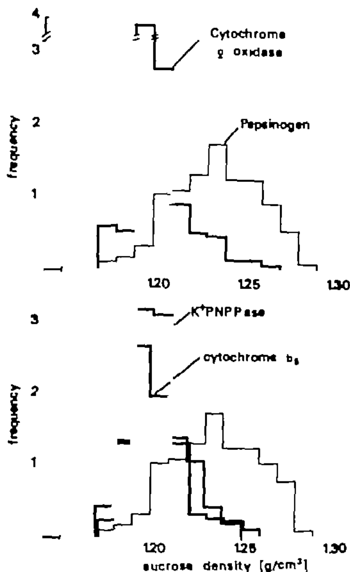


Fig 2: Isopycnic equilibration of isolated rat fundic cells in linear sucrose gradient. Standardized representation according to ref 5. The term Frequency on the ordinate axis refers to dQ/Q dρ, dQ standing for the fraction of the total activity (Q) recovered and dρ for the elementary density step

(a) Parietal cells marked by cytochrome c oxidase are distinctly separated from other cell types here illustrated by pepsinogen (mucous and peptic cells behave identically)

(b) K^+ PNPPase and cytochrome b_5 equilibration profiles are closely similar to that of cytochrome c oxidase and clearly distinct from that of pepsinogen (thin line) hence they do relate to parietal cells

Parietal cells were also shown to be enriched in HCO_3^--stimulated SCN^--inhibited ATPase This enzyme has been for some time suggested to be critically involved in H^+-transport (30 31) Previous report from this laboratory has however provided strong evidence for its mitochondrial derivation and explained its presence in purified gastric plasma membranes in terms of mitochondrial contamination (13) This was recently confirmed (32) Thus it may appear that enrichment of parietal cells in HCO_3^--stimulated ATPase is but a simple consequence of their peculiar wealth of mitochondria

Finally although Golgi apparatus is only occasionnally observed in the parietal cells the latter were found to display a substancial galactosyl transferase activity

Subcellular origin of cytochrome b_5 and K^+ PNPPase in the parietal cell.

Homogenates prepared from isolated rat fundic cells exhibited the same enzymatic pattern as those prepared from whole scraped fundus Since in particular the value for K^+-PNPPase and cytochrome b_5 were very similar respectively in both preparations it may be assumed that cells isolation procedure, and specially the use of pronase for tissue dissociation has no sequel on these components (table)

COMPARISON BETWEEN GASTRIC HOMOGENATES

	Scraped fundus	Isolated cells
Pepsin (1)	1 5	1 3
Cytochrome c oxidase (2)	6 4	6 3
K^+-ATPase (3)	ND	ND
K^+-PNPPase (3)	16 8	14 6
Cytochrome b_5 (4)	28 6	28 8

Units per mg protein (1) µmoles tyrosine min^{-1}; (2) Δ log DO min^{-1}; (3) nmoles min^{-1}; (4) pmoles
ND no detectable activity

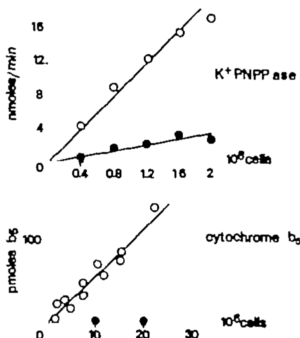


Fig 3: *K^+ PNPPase activity and cytochrome b_5 content per 10^6 isolated gastric cells (mixed population) as a function of cell concentration for intact (close circles) as compared to disrupted (sonicated) cells (open circles)*

Supportingly since cell disruption was required for full determination, K^+-PNPPase and cytochrome b_5 are strongly suggested to be endocellular (fig 3)

Homogenates were submitted to differential centrifugation and subcellular fractions thus obtained were characterized for usual biochemical markers of membrane structure and for cytochrome b_5 content and K^+-PNPPase activity (fig 4) Cytochrome b_5 exhibited a typical microsomal distribution profile roughly similar to that of 5 AMPase (with however a lack of cytochrome in the supernatant fraction) with a maximal enrichment in (P) fraction (x 3 5 in terms of RSA) K^+-PNPPase although it was highly enriched in (P) (x 3 4) had however a distribution slightly shifted towards larger sized microsomes with a maximum enrichment (x 3 6) in (L) fraction Both components were clearly distinct from mitochondrial marker such as cytochrome c oxidase

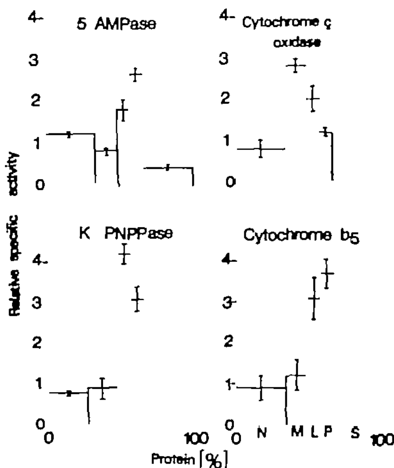


Fig 4: *Subcellular localization of K⁺ PNPPase and cytochrome b5 differential centrifugation of gastric homogenates (see Material and Methods) mean value of 3 5 experiments _ 1 SE with recoveries ranging from 90% to 110%*

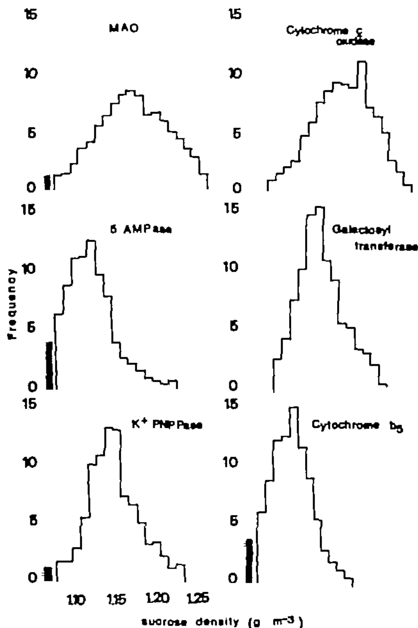


Fig 5: Subcellular localization of K^+ PNPPase and cytochrome b_5; isopycnic equilibration of gastric membranes (fraction (L P) see Material and Methods) Representation standardized as in fig 2 of one typical experiment

(L) and (P) fractions were then pooled and applied to a linear sucrose density gradient Cytochrome b_5 and K^+-PNPPase equilibrated with a peak buoyant density of 1 105 g cm^{-3} and 1 113 g cm^{-3} respectively Their distribution profiles were clearly dissociated from that of both cytochrome c oxidase and monoamine oxidase which thus fully confirming their non-mitochondrial origin These profiles were by contrast rather typical of smooth microsomal membrane components Close to that of 5 AMPase they were however markedly distinct from that of Galactosyl transferase (fig 5)

These findings are in good agreement with previous data reported for gastric mucosa of other species (6 7 9 23 27) They suggest that in the parietal cell cytochrome b_5 and K^+-PNPPase may be located on plasma membrane and/or smooth endoplasmic reticulum

Further resolution of smooth membranes by the use of digitonin.

As previously shown in liver (12 33) digitonin was found to form stoechiometric stable complex with cholesterol bound to gastric membrane with a resulting specific shift of cholesterol-rich membranes equilibration profile towards higher densities

This digitonin shift was successfully used in liver to separate plasma membranes (assumed to be rich in cholesterol) from smooth endoplasmic reticulum membranes (assumed to contain very little if any cholesterol) On gastric microsomes digitonin binding resulted in a dramatic displacement of equilibrium density of both 5 AMPase and K^+-PNPPase (+ 0 049 g cm^{-3}); equilibrium density of cytochrome b_5 was however almost unaffected (fig 6) According to the data in liver such a finding in the parietal cell would suggest that K^+-PNPPase does belong to plasma membrane compartment while cytochrome b_5 should be actually just like in hepatocyte (12 34) a specific marker of smooth endoplasmic reticulum

With consideration to the morphological features of the parietal cell it is very tempting to infer from the above

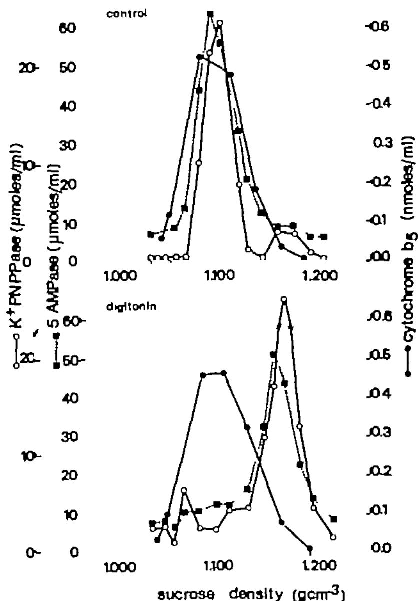


Fig 6: Effect of digitonin treatment on equilibrium density of gastric membranes In control (upper graph) cytochrome b_5 equilibrium density is very close to that of 5 AMPase and K^+ PNPPase After digitonin treatment (lower graph) the latter markers are selectively shifted to the right presumably because they belong to membranes rich in cholesterol.

results that *ipso facto* cytochrome b_5 should be a marker of tubulovesicles If this were true then it would be admitted that K^+-PNPPase should not be associated with the tubulovesicles (but rather associated with apical plasma membrane ?)

However it may be alternatively proposed that by contrast with hepatocytes smooth endoplasmic reticulum membranes in parietal cells contain as much cholesterol as plasma membranes do and therefore cannot be discriminated from the latter on the basis of the digitonin shift model Such a common feature between these two membrane compartments would be consistent with their suggested ability to fuse one with another In this alternative K^+-PNPPase could be proposed as marker for tubulovesicles with cytochrome b_5 being associated with other undefinite smooth endoplasmic membranes (distinct from Golgi apparatus) This should be in agreement with the finding that although they behaved almost similarly in subcellular fractionation microsomes bearing K^+-PNPPase and those bearing 5 AMPase however differed slightly in terms of size and density (above results) and markedly in terms of electric charge (9)

Finally it may be also proposed that the tubulovesicular system is actually heterogeneous in nature with two vesicles subpopulations distinctly marked with cytochrome b_5 and K^+-PNPPase respectively

Conclusion

Thus the data presented in this study clearly demonstrate in the rat fundic mucosa the specificity of both K^+-PNPPase and cytochrome b_5 for the parietal cells Since these cells may appear to be exclu ively devoted to H^+-transport such a finding strongly argues for a role more or less direct of K^+ PNPPase and cytochrome b_5 in this transport This is furthermore supported by the suggested location of these components on subcellular structures relating to plasma membrane and/or tubulovesicular system (these structures should be however distinct for K^+-PNPPase and cytochrome b_5) However since

at variance with previous reports by others we cannot find in our hands any K^+-ATPase activity it seems difficult at the present time to schematize the findings in a comprehensive functional model

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Role of K^+ stimulated ATPase in H^+ and K^+ Transport by Bull Frog Gastric Mucosa *in vitro*

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ABSTRACT Resting and spontaneously secreting mucosa secrete K^+ at a steady rate Histamine (0 2mM) stimulated both H^+ and K^+ secretion with the peak for H^+ following that for K^+ The latter returns to the non stimulated level within 45 min while the H^+ secretion remains at a high steady state level The pCMBS which completely inhibited the K^+ stimulated ATPase and pNPPase associated with the purified light microsomal fractions from the bull frog oxyntic cells also decreased H^+ secretion to zero and allowed the K^+ level to increase to a constant higher level at 60 min Substitution of pCMBS with 2mM 2 mercaptoethanol in the mucosal solution completely restored the H^+ secretion and returned the K^+ value to the non-stimulated level The pNPPase activity associated with the mucosal surface showed parallel responses with the H^+ secretory state of the tissue

The physiological implications of K^+ in the gastric juice is not understood although it has been well extablished in the past decades that this ion may play an important role in gastric secretion (1 3) It has been demonstrated that K^+ in the nutrient solution (4–6) is absolutely necessary for the maintenance of acid secretion by bullfrog gastric mucosa in vitro Recently a K^+ stimulated ATPase have been localized on the apical region of the parietal cells (8) and the membrane vesicles derived from these apical plasma membranes are capable of producing a H^+ gradient in the presence of ATP and K^+ (9 10) These discoveries strengthened the idea that K^+ plays an important role in gastric acid secretion However the precise relationship existing between K^+ and H^+ transport under physiological conditions still remains unclear The data presented in this paper suggests the following a) there are distinctly different routes for the transport of H^+ and K^+ by bull frog gastric mucosa and an intimate relationship exists between the two and b) the K stimulated ATPase is involved in the transport of protons and during this process K is transported back inside the cell from the lumen

by the ATPase system These results are discussed in terms of a hypothetical mechanism for gastric HCl secretion

METHODS

A Transport studies

The fundic mucosa from Rana Catesbeiana was carefully separated from the submucosa and mounted over one end of a plastic tube (13x100 mm) with the mucosal surface (M) facing out The nutrient solution (N) wa a regular frogs Ringer (F R) solution (11) which was equilibrated with 95% O_2 5% CO_2 The mounted tissue was placed in a thermostated Radiometer titration vessel which was maintained at 37°C with an external circulating water bath The mucosal surface (M) was bathed with 10 ml 104 mM NaCl The mucosal solution was gassed with 100% O_2 and stirred continuously with a magnetic stirrer The mucosal solution was collected at 15 min intervals and placed in thoroughly washed plastic vials The K^+ content of the secretory medium was determined flame photometrically and the H^+ secretion was quantitated by titration with 1 mM NaOH to pH 6 5 while gassing with 100% N_2

B Surface p-nitrophenyl phosphatase (pNPPase) assay

The surface pNPPase was assayed following the method of Durbin and Kircher (11) The mucosal solution consisted of 87 mM NaCl 18 mM $NaHCO_3$ and 0 5 mM pNPP Both nutrient and mucosal solutions were bubbled with 95% O_2 5% CO_2

C Gradient purified membranes

The membranes enriched in K^+ stimulated ATPase and pNPPase were prepared according to the method of Ray and Forte (12) with the following modifications The homogenizing medium consisted of 250 mM Sucrose con taining 0 2 mM EDTA and 2 mM PIPES pH adjusted to 6 8 The purified light membrane fraction was harvested from the post nuclear supernatant by centrifugation for 4 hours at 100 000xg on a discontinuous sucrose (37%) gradient

D Enzyme assays

The K^+ stimulated ATPase and pNPPase was assayed by the methods des cribed previously (7) Assay of the phosphorylated intermediate has been described (13)

E Statistical treatment

Paired student t test was used to compare secretory data obtained in

test condition with initial steady state control values for the same mucosa and the differences were regarded as significant when $P<0\ 05$ The variability of samples is expressed as the mean $\pm$ SE

RESULTS

Fig 1 shows the relationship between the appearance of H^+ and K^+ in the secretory fluid after histamine stimulation As can be seen in this graph histamine stimulated a sustained secretion of H^+ (1 20 µEq/hr to 4 40 µEq/hr after histamine stimulation) and a transitory efflux of K^+ (about 2 76 µEq/hr) which returns to the control level of 1 75 µEq/hr after about 45 minutes The peak for K^+ preceeded that for H^+

This pattern of appearance of K^+ in the secretory fluid can be ex plained in two ways Histamine (secretagogue) may cause some transient change in permeability of the secretory membranes to K^+ This altered permeability of the secretory membrane to K^+ then may be gradually re stored to its original state The K^+ efflux may or may not have any re- lation to the H^+ transport The second possibility is that the

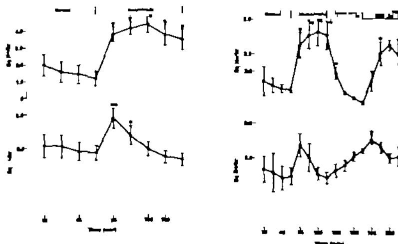


Fig 1 (Left) Effects of histamine on H^+ and K^+ transport by bull frog gastric mucosa in vitro The spontaneously secreting mucosa was washed thoroughly in F R solution with several changes and then mounted in a chamber Details are given in METHODS
$P<05$, $^{**}P<01$ n=12

Fig 2 (Right) The effects of sequential addition of 0 25 mM p-chloro-mercuribenzene sulfonic acid (pCMBS) and 2mM 2-mercaptoethanol (BME) in the mucosal solution (M) of the chambered mucosa stimulated with 0 2mM histamine H^+ transport (upper graph) and K^+ transport (lower graph) are shown as a function of time n=6

secretagogue stimulated mucosa may have a sustained higher rate of K^+ efflux and this observed decrease in K^+ efflux after the initial transient peak may be due to recycling of K^+ back into the cell There may exist some relation between the transport of H^+ and recycling of K^+

In order to distinguish between these two possibilities we exposed the histamine stimulated mucosa to pCMBS on the mucosal side to inhibit H^+ transport and monitor K^+ transport under these conditions It has been shown previously by Solberg and Forte (14) that in bull frog gastric mucosa pCMBS completely inhibits H^+ transport which can be completely reversed by 2 mercaptoethanol (BME) a thiol agent Our result confirms that of Solberg and Forte and in addition shows the pattern of K^+ efflux as shown in Fig 2 During histamine stimulation the sulfhydryl agent pCMBS (0 25 mM) added to the secretory solution reduces the acid secretion to zero within 45 minutes and caused a rapid increase in K^+ efflux which began stabilizing at 50 minutes post pCMBS addition Replacement of pCMBS by BME a thiol agent resulted in a small transient increase and then a rapid decrease in K^+ output. This decrease in K^+ output was coincidental with an increased H^+ secretion These results clearly demonstrated that in frog gastric mucosa there is a pathway for the exit of K^+

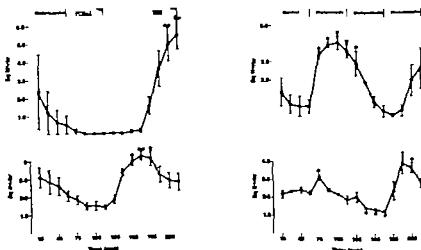


Fig 3 (Left) Effects of sequential additions of pCMBS histamine and BME on the metiamide pretreated resting mucosa pCMBS was replaced with BME in the mucosal solution (M) while histamine was in the nutrient (N) solution n=3

Fig 4 (Right) Effects of histamine and metiamide on H^+ and K^+ transport by the chambered mucosa The order of addition of the agents are shown in the upper part of the graph n 3

which is independent of H^+ transport The enhanced rate of K^+ output after pCMBS treatment may be due to either inhibition of K^+ recycling by pCMBS or a direct effect of pCMBS on the K^+-conductive pathway If the K^+ recycling is inhibited by pCMBS which also inhibits H^+ transport it is very likely that both the processes are operated by a common transport machinery

To distinguish between the possibility of a direct effect of pCMBS on K^+ conductive pathway and the K^+ recycling pathway through the H^+ transport machinery we did the following experiment We added pCMBS to the secretory solution in a metiamide pretreated resting mucosa There was no significant change in either H^+ secretion or K^+ efflux However when the resting mucosa with pCMBS on the secretory side was stimulated with histamine there was a three fold increase in K^+ efflux ($p<0.05$) with no significant change in H^+ secretion While maintaining histamine stimulation replacement of pCMBS with BHE resulted in a significant increase in H^+ secretion ($p<0.02$) with consequent reduction of K^+ efflux which was not significantly different than that of metiamide alone These results shown in Fig 3 demonstrate that pCMBS itself does not have any significant effect on the K^+ conductive pathway Therefore we may conclude that pCMBS which inhibits the H^+ transport also inhibits the K^+ recycling process and the effects are possibly mediated through the same transport machinery

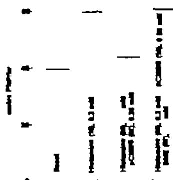
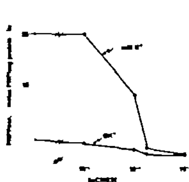


Fig 5 Effects of various agents added into the nutrient (N) and mucosal (M) solutions on the pNPPase activity of the secretory surface membranes Mucosal samples were collected 60 min after addition of the agents

Since Metiamide was used in this experiment to bring the mucosa to resting state it was important to see the effects of histamine alone on the metiamide pretreated resting mucosa with respect to the H^+ and K^+ transport parameters. The results are shown in Fig 4. The general pattern of the H^+ and K^+ transport characteristics of the mucosa remained unchanged. However, the metiamide caused a small but significant decrease in K^+ efflux from the nonstimulated control level.

K^+-ATPase, recently localized on the secretory membranes of the parietal cells has been strongly implicated to be involved in H^+ transport. The activity of a surface pNPPase located on the secretory surface of the bull frog gastric mucosa has been demonstrated by Durbin and Kircher (11) to show a good correlation with the secretory capacity of the tissue. If this surface pNPPase is a manifestation of the K^+ ATPase, which is thought to be the transport machinery for H^+, we should be able to monitor the H^+ transport capacity of the mucosa using the surface pNPPase as an indicator. The results of such an experiment is shown in Fig 5. Although the spontaneously secreting tissue shows a high pNPPase activity (designated as "basal" here), it does show the expected parallel response with the various agents. Thus histamine stimulation increased, pCMBS treatment decreased and BME treatment returned the surface pNPPase activity to the original histamine stimulated level.

Purified membrane fractions enriched in the K^+ ATPase and K^+ pNPPase were isolated from the oxyntic cells of bull frog gastric mucosa to test



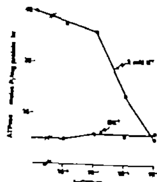


Fig. 6. (Left) Effects of increasing concentrations of pCMBS on K^+ stimulated pNPPase of gastric microsomes.
Fig. 7. (Right) Effects of increasing concentrations of pCMBS on K^+ stimulated ATPase of gastric microsomes.

the effects of pCMBS on those enzymes Fig 6 shows the effects of increasing concentrations of pCMBS on the K^+ pNPPase of the purified gastric microsomes The enzyme activity is completely inhibited at 0 25 mM pCMBS the same concentration used in our chamber study Fig 7 shows the effects of pCMBS on the K^+ ATPase activity Compared to pNPPase the K^+ ATPase was completely inhibited at a much lower concentration of pCMBS This differential inhibitory effect of pCMBS on K^+pNPPase and K^+ ATPase suggest easy accessibility and possibly higher affinity of that agent for the ATPase catalytic site in the isolated vesicles However in intact tissue probably pCMBS can not permeate the cell membrane and bind with the catalytic site of the ATPase molecule since even after 60 minutes exposure of the secretory surface with 0 25 mM pCMBS the inhibitory effect of H^+ transport is readily reversible by BME

Since the K^+-ATPase was more sensitive to pCMBS inhibition than K^+ pNPPase it would suggest that the primary action of the pCMBS would be at the level of formation of the 32 p-intermediate of the ATPase reaction Studies on the phosphorylated intermediate of the K^+ ATPase confirms this prediction Results are shown in Fig 8 As expected pCMBS treatment produces much lower level of 32 p-intermediate compared to the control and the phosphorylated intermediate of the pCMBS treated membranes is completely insensitive to K^+

DISCUSSION

The rapidity of the pCMBS action on H^+ transport and its ready reversibility with 2-mercaptoethanol suggests that it acts at or very near the

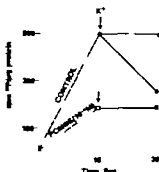


Fig 8 Effects of K^+ 2mM and pCMBS on the phosphorylated intermediate(s) of the K^+ ATPase associated with gastric microsomes Control membranes without K^+(-O-) with K^+ (●) pCMBS pretreated membranes without K^+ (-□) with K^+ (-■)

outer surface of the apical membrane presumably at a site responsible for transport of protons Very poor permeability of pCMBS through the membranes has been demonstrated in a variety of cells (15-17) including bull frog gastric mucosa (14)

pCMBS when applied to the secretory side of the histamine stimulated mucosa inhibits specifically the H^+ transport the K^+ transport being enhanced under this condition (Fig 2) These data suggest that there are two distinctly different routes for the transport of H^+ and K^+ from the cell into the lumen

Under conditions such as histamine stimulation (Fig 1 and 2) substitution of pCMBS by 2-mercaptoethanol (Fig 2) and replacement of metiamide by histamine (Fig 4) an exodus of K^+ from the cell into the lumen occurs *which preceeds the H^+ transport These observations suggest a casual relationship* existing between the K^+ and H^+ transport by gastric cells Secretagogues are believed to act by mobilization of substrates for oxidative metabolism which is necessary to meet the energy demands during HCl transport However in view of our experimental data showing K^+ transport preceeding H^+ transport it is tempting to speculate that secretagogues such as histamine may also influence H^+ secretion by regulating the K^+ transport

The K^+ stimulated ATPase located on the apical membranes of the parietal cells has been strongly implicated in the H^+ transport process (8) Furthermore using radiolabeled iodination technique it has been shown that the K^+ stimulated ATPase molecule with its glycoprotein submit spans the whole membrane bilayer (18) Since pCMBS inhibits H^+ transport

Fig 9 A proposed model for the mechanism of H^+ transport by oxyntic cells of bull frog gastric mucosa E stands for the K^+ stimulated ATPase and E~p 2 for the phosphorylated intermediate(s) of the enzyme

from the lumen side (Fig 2) and completely inhibits the K^+ stimulated ATPase and pNPPase in vitro (Fig 6 and 7) it is highly likely that this agent inhibits the transport enzyme in the isolated mucosa by binding to that portion of the molecule which extends out into the lumen at the secretory membrane

Recent studies on the mechanism of K^+ stimulated ATPase (13) and the transport studies with isolated gastric microsomal vesicles (9 10) suggests the feasibility of this K^+ stimulated ATPase in K^+ H^+ exchange process The data presented in this paper also supports this hypothesis Thus in the presence of pCMBS which inhibits strongly the K^+ stimulated ATPase the recycling of K^+ from the lumen into the cell would stop Under this situation one would expect an increase in the out flux of K^+ The results shown in Fig 2 and 3 demonstrate an increase in the rate of appearance of K^+ in the secretory solution after pCMBS treatment However for any such recycling of K^+ the transport pathway for K^+ should be in close juxtaposition to that of the K^+ stimulated ATPase molecule which is believed to be involved in the proposed recycling process Such close association in molecular architecture of the transport molecules would allow a majority of the K^+ to be recycled back into the cell interior instead of escaping into the secretory fluid during acid secretion

Our data is consistent with a model (Fig 9) where the secretory membrane contains a K^+ conductive pathway and a K^+ stimulated ATPase system The former is responsible for diffusion of K^+ from the cell into the lumen and the later is responsible for recycling of K^+ with the resultant deposition of H^+ into the lumen Thus pCMBS which inhibits the K^+ ATPase and K^+ pNPPase would not only stop the transport of protons but also stop the recycling of K^+ As a result one would expect to see an enhancement in the efflux of K^+ in the secretory solution This is exactly what we find in the in vitro frog gastric mucosa However it should be cautioned that this is a simplified and highly schematic representation based on some fundamental assumptions Thus although our data suggest that there is a separate pathway for the transport of K^+ from the mucosa into the lumen it does not tell us anything about the cell type In this scheme we have assumed that the K^+-conductive pathway is located at the secretory membrane of the parietal cells It has also been assumed that the surface pNPPase activity of the intact mucosa is a manifestation of the K^+ stimulated ATPase an assumption which needs to be

proved Furthermore this model has been proposed on the basis of our study with histamine in isolated bull frog gastric mucosa Therefore generality of the proposed scheme remains to be tested using other secretagogues such as pentagastrin acetylcholine etc in various species

ACKNOWLEDGEMENTS

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Tissue and Cell Localization of Hog Gastric Plasma Membrane by Antibody Technique

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ABSTRACT Antibodies have been obtained which specifically interact with the transport enzyme ($H^+ + K^+$)-ATPase The antigen used was a highly active gastric plasma membrane preparation from hog fundic mucosa purified to homogeneity as judged by polyacrylamide gel electrophoresis Gamma globulin from immunized animals but not from control animals or preimmunesera inhibited the K^+-ATPase activity up to a maximum of 80% Incubation of alcohol fixed parafin embedded section of hog fundus and antrum with immune and preimmunesera as well as isolated rat parietal cells followed by fluorescein conjugated anti rabbit γ-globulin showed specific binding of the latter (1) only following immunesera (2) only in the fundus (3) only in the parietal cells (4) mainly in the apical region These data confirm that the site of origin of the ($H^+ + K^+$) ATPase vesicles derives from the gastric parietal cell and is compatible with a role of this system in gastric H^+ secretion

Over the past several years the complex mechanism of gastric acid secretion by the gastric mucosa has been the subject of much research through electrophysiological (1 2 3) metabolic (4 5 6) and ultra structural studies (7 8 9 10) of this heterocellular epithelium

To date it seems well established that the acid secretory cell of the gastric mucosa contains an elaborate system of smooth membranes (tubulovesicles) which are abundant in the apical region of the parietal cell and are directly involved in the secretory process (11 12 13)

Evidence has previously been presented to support the suggestion that the tubulo-vesicular membranes are isolated with a purified gastric

microsomal fraction derivated from fundic homogenate either from amphi bian or mammalian tissue (14 15 16 17)

Such purified microsomes possess an ATP hydrolyzing system perhaps unique for the gastric tissue which is cation dependent and is capable of catalyzing a K^+ H^+ exchange across the vesicle membranes and to act as an electroneutral proton pump (18 19)

Based on these findings it is important to define the cell type and the region of the cell from which the active fraction originates In the present work immunological techniques were used for this purpose

MATERIALS AND METHODS

1 Membrane Isolation Gastric membranes from hog fundic mucosa were prepared as previously described (17) by differential centrifugation density gradient separation on zonal rotor and free flow electrophoresis The final three fractions designated FI (vesicular structure associated with K^+-ATPase and K^+ pNPPase activities) FII (membranes containing the basal Mg^{2+}-ATPase and 5 -nucleotidase) and FIII (essentially membrane fragments containing K^+ ATPase and pNPPase activities) were routinely lyophilized and stored at 80 C prior to use for antibody study

2 Preparation of antisera to membrane protein: New Zealand white rabbits were pre bled and sera were obtained after clotting at r t for 1 hour and overnight at 4 C then centrifuged at 3 000 x g for 15 minutes

Two weeks after pre bleeding the rabbits were immunized using 1 mg of FI membrane protein fraction suspended in 1 ml of 0 9% NaCl solution and mixed with an equal volume of Freund s complete adjuvant

Subsequent injections were administered bi-weekly for eight weeks and five days after the second injection the rabbits were bled and sera pre pared as described above Thereafter rabbits were bled weekly and sera tested for immunreactivity towards the respective antigen (membrane pro-

tein) by double immunodiffusion (20)

3 Fractionation and purification of antisera Preparation of γ- globulins from preimmunesera as well as antisera were carried out by drop-wise addition at 4 C of a volume of saturated $(NH_4)_2SO_4$ solution equal to the volume of the sera After centrifugation at 10 000 x g for 30 minutes the precipitate was resuspended in a volume of deionized water equal to 50% of the original volume of the sera The precipitation and resolubilization cycle was repeated three times

The final γ-globulin fractions were dialyzed against 150 mM Tris Cl buffer (pH 7 4) at 4 C for a period of 72 hours

In all experiments the antibody fractions were further purified by absorption on insoluble antigens (21) Antibodies and preimmunesera aliquots of about 0 5 ml each were stored at 20°C until use

4 Chemical determination Protein was determined by the method of Lowry et al (22) ATPase 5 -nucleotidase and pNPPase were analyzed as previously described (17)

For experiments in which the effect of antibodies on enzyme activities was studied antigens either fresh or lyophilized were pre incubated at 22 C for 30 minutes with the purified antibodies and preimmunesera as controls in the assay medium before substrate addition

5 Cell isolation from rat fundic mucosa Cell isolation and separation was performed following the method of Lewin et al (23) Separation of parietal cells from other cell types was achieved by a serial centrifugation technique carried out at 100 x g for 45 seconds (24)

Parietal cells were identified by the dark blue granules after succinic dehydrogenase stain using nitrotetrazolium blue (25)

6 Immunohistochemical studies Immunofluorescence technique was carried out using the method of Sainte-Marie (26) Fifty to 100 µl of

antibodies (1 5 to 3 0 mg of protein) were applied to 5 6μ thick sections and incubated in moist atmosphere at r t for 45 minutes

After washing in phosphate-buffered saline the sections were stained by indirect method (27) and fluorescein isothiocyanate conjugated goat anti rabbit γ-globulin (Hyland Lab Los Angeles CA) reconstituted in 1 0 ml of sterile distilled water using 50 100 μl of a 1/10 dilute solution

Following incubation at r t for 45 minutes the sections were washed overnight and mounted in buffered ethanol Stained sections were examined with a Leitz fluorescence microscope and photographed The specificity of the staining was determined by using the preimmunesera and rabbit anti IgG IgA and IgM as controls

For the immunofluorescence study enriched preparations of rat parietal cells were washed twice in phosphate-buffered saline containing 1% bovine serum albumin and 0 03% Na azide Two hundred μl of cell suspension containing about 1 5 x 10^6 cells were incubated at r t for 1 hour with 100 μl of antibodies or γ globulin from non immunized rabbit

After being spun down at 100 x g for 5 minutes the sediment was washed twice The pellet was resuspended in 200 μl of phosphate buffered saline and 100 μl of 1/10 dilute fluorescein conjugated goat anti rabbit γ globulin were added After 30 minutes incubation at r t the cells were washed and the final pellet dispersed in a few drops of buffer The suspension was examined under the fluorescence microscope

7 Polyacrylamide gel electrophoresis Gel electrophoresis was performed with 5 6% acrylamide (28) in 0 1% SDS Samples were solubilized in 1 0% SDS 1 0% β-mercaptoethanol and 10 15 μg of protein were applied to each gel Staining and distaining for protein and carbohydrates were carried out as previously described (16)

RESULTS AND DISCUSSION

The gastric K^+ ATPase is a membrane bound enzyme composed of about 95% of one glycoprotein of 105 000 M W It has been purified from hog fundic mucosa (17) using differential centrifugation density gradient separation and free flow electrophoresis The fractionation procedure yielded a distinct separation between membranes carrying Mg^{2+}-ATPase and 5 -AMPase and membranes containing K^+-ATPase (or p-nitrophenyl phosphatase)(Table I)

TABLE I

PURIFICATION OF GII GASTRIC MEMBRANE FRACTION
Activities are expressed in µmoles Pi and pNP/mg protein/hr

Fractions		5 -AMPase	Mg^{2+}-ATPase	K^+-ATPase	K^+-pNPPase
GII	(zonal)	2 2 + 0 3	19 4 ± 1 8	63 6 ± 2 0	61 2 ± 4 2
GII FI	(FFE)	0 3 ± 0 06	4 4 ± 1 0	110 7 ± 2 6	68 0 + 2 5
GII FII	(FFE)	5 4 ± 1 0	31 0 ± 3 1	20 0 ± 0 7	9 4 ± 1 0
GII FIII	(FFE)	0 1 ± 0 1	2 8 ± 0 4	50 1 + 3 2	31 0 + 2 2

*Activity in presence of 20 mM KCl minus the basal rate

Along with the 40-fold enrichment in K^+-ATPase activity (17) this vesicular fraction FI responded to ATP addition in terms of H^+ uptake and K^+ extrusion (18 19)

Although ultrastructural evidence indicates that the parietal cell contains an abundant intracytoplasmic vesicular system (11 12) and H^+ transport is a property of the isolated FI vesicles (18 19) direct evidence that these vesicles derive from the acid secretory region of the parietal cell has been lacking

Since enzyme-specific antibodies have proven to be sensitive and powerful probes for the purpose of elucidating structural and functional characteristics of certain enzymes (29 30 31) as well as their locali

zation into the particular biological system from which they derive (32) we used immunochemical studies directed to determine the tissue and cell origin of the purified K^+ ATPase membrane fraction

Antibodies were obtained from the serum of rabbits injected with several preparations of purified and highly active native enzyme The γ-globulin obtained from anti serum was purified by adsorption to and elution from the enzyme in the native membrane fraction and the antigen antibody reaction was tested by double diffusion technique

Immunodiffusion of purified anti K^+ ATPase γ globulin (FI AB) against Triton X 100 solubilized enzyme (FI Ag) gave single precipitation band while no immunoreactivity was detected when FII and FIII membrane fractions were used (Fig 1) Serum and γ globulin from non immunized rabbits did not give any precipitation bands

These results indicate that specific complex formation is only obtained between FI Ag and FI AB

It is known that antibodies may produce inhibition or stimulation of enzyme activity may alter certain physical characteristics or may react with the enzyme without causing detectable changes in enzymatic activity (31)

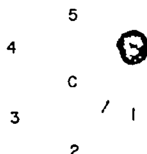


Fig 1 Double diffusion gel precipitation Well C contains FI AB Peripheral wells contain (1) FI Ag (2) FII Ag; (3) FIII Ag (4) control globulin (5) control globulin Antigens were solubilized in 1% Triton X 100

Fig 2 Inhibitory effects of varying concentration of FI AB preparation on K^+-ATPase and pNPPase activities In these studies 5 µg of enzyme were used

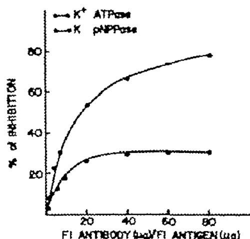


Fig 2 shows the effects of varying concentration of the purified anti K^+-ATPase γ-globulin obtained from two different rabbits on K^+ ATPase and pNPPase activities of the native enzyme It is evident from the results that the degree of inhibition of enzyme activities was proportional to the increasing amounts of antibody with maximal inhibition of about 80% for the K^+ stimulated ATPase and approximately 30% for the K^+ activated pNPPase The control γ-globulin fraction from non immunized rabbit did not inhibit the enzyme activities Since no inhibition of the basal Mg^{2} -ATPase was detected this antibody is specific for the K^+ activation step consistent with its effect on the phosphatase

However antibodies obtained from two other rabbits did not affect the enzyme activities although showing immunreactivity when tested by double-diffusion technique against solubilized FI Ag

Since in this case the antibodies did not inactivate the enzyme activity it was necessary to provide convincing evidence that they were bound specifically to the enzyme and not to impurities For this purpose FI Ag in native form was incubated with different concentrations of FI AB at 37 C for 30 min The mixtures were allowed to stand at 4 C



Fig 3 SDS polyacrylamide gel electrophoresis (FI) purified K^+-ATPase membrane fraction (AB) FI specific antibody (C) FI Ag FI AB complex Experiments were carried out as described in the text

overnight and were then centrifuged at 100 000 x g for 60 min After the pellets were washed by resuspension in water followed by centrifugation for three times the final immunoprecipitates were dissociated in 1% SDS and electrophoresed as described (28)

Fig 3 shows the gel pattern obtained from a typical experiment It can be seen that the immunoprecipitate contains only two polypeptides corresponding to FI Ag and FI AB indicating a specific non-covalent binding between the two proteins When FII membrane fraction was incubated with FI AB and experiments carried out as described above the gel pattern of the immunoprecipitate showed only the presence of FII poly peptides pattern indicating once more that FI AB was specifically produced against FI Ag This is in agreement with the fact that greater than 95% of the protein in the immunizing FI Ag was K^+ ATPase

The availability of antibody specifically directed against the membrane bound K^+-ATPase made it possible for us to determine the localization of

this antigen and its specificity among different tissues

Indirect immunofluorescence technique was applied on hog fundic sections using the purified anti K^+-ATPase The staining pattern illustrated in Fig 4 shows that the majority of the fluorescence is localized in the middle third region of the gastric gland where the majority of parietal cells is present. This strong fluorescence appears also to have mainly a supranuclear apical region distribution which corresponds to the region of the tubulo vesicles or microvilli of the parietal cell

Sections treated with γ-globulin obtained from sera of non immunized rabbits were uniformly negative Antrum sections as well as sections from ten other different tissues did not show any positive fluorescence staining confirming the specificity of the FI AB only for the fundic mucosa

It is often difficult to determine with accuracy the cell type and cell region from which a particular antigen derives just by fluorescent tissue sections especially when it is a complex system like the gastric mucosa Immunofluorescence studies performed on isolated and separated cells from fundic mucosa could be more informative

When suspensions of isolated cells from rat stomach were treated with



Fig 4 Indirect immunofluorescent staining of hog fundic mucosa gland in the middle third region with FI AB (x270)



Fig 5 Staining pattern observed in rat isolated parietal (right) and chief (left) cells with FI AB (x775)

purified anti K^+-ATPase antibody and goat anti rabbit γ-globulin fluorescent reagent only the parietal cells shows a strong positive staining (Fig 5) On some parietal cells of the same preparation fluorescence was distributed on the entire surface while on most of them a more regular perimeter membrane staining was observed The intensity of the stain was quite pronounced in one region of the cell probably the apical surface (Fig 6) Suspensions treated with γ-globulin from non immunized rabbit were uniformly negative

In conclusion we have demonstrated that antibody raised against and



Fig 6 Immunofluorescent staining of an isolated rat parietal cell with FI anti K^+ ATPase (x775)

specific for a gastric K^+ ATPase membrane system stains by immunofluorescence cells derived from fundic epithelium and does not stain cells originating from other tissues Thus this antibody appears to be organ specific

In addition the antibody may well be cell type-specific because it does not stain cells other than parietal The antigen detected by the anti K^+-ATPase antibody is located on the parietal cell surface as demonstrated by the perimeter type of immunofluorescence staining

Because viable intact cells were used in the present experiments rather than fixed cells the possibility that the surface antigen detected by anti K^+-ATPase is also present in the intracytoplasmic vesicular system cannot be ruled out

The recent technique of ultracryotomy (33) for locating antigens and determining their distributions within cells ultrastructural intact by staining them with ferritin-conjugated antibodies will definitely prove this point Such studies are presently in progress

ACKNOWLEDGMENT

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Stimulation of Endogenous Cyclic AMP (cAMP) in Isolated Gastric Cells by Histamine and Prostaglandin

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ABSTRACT: Suspensions of isolated mucosal cells from rat corpus of high and low parietal cell content were prepared They were stimulated by theophylline given alone or together with histamine histamine plus cimetidine diphenhydramine or mepyramine or prostaglandin E_1 Cellular cAMP concentration was determined by radioimmunoassay Theophylline stimulated cAMP levels were similar in all pools In contrast histamine stimulated the pools with a high parietal cell content more than the pools with a low content Prostaglandin E_1 increased cAMP concentration more in pools with a low parietal cell content than in pools with a high parietal cell content A statistically significant linear regression between stimulation and parietal cell content was obtained in both cases Histamine action was inhibited by low concentrations of cimetidine but only by very high concentrations of histamine-1 receptor antagonists It is concluded that histamine stimulates cAMP formation exclusively in parietal cells and that it exerts its effect via an histamine-2 receptor Prostaglandin E_1 may inhibit cAMP formation of parietal cells In addition it stimulates cAMP formation in mucosal cells different from parietal cells

INTRODUCTION

Since the fundic mucosa of the stomach contains different cell types timulus secretion coupling is difficult to assess (1 4 8 9) In the intact mucosa both stimulants and inhibitors of gastric acid secretion stimulate cAMP formation Similar observation were made in membrane preparation of gastric mucosa (2 5 12 13 18 19 20) Therefore it is not known whether cAMP is formed in parietal cells and whether it

mediates acid secretion

In the present study isolated rat fundic mucosal cell suspensions were divided into fractions containing different cell types It was shown that cAMP is formed in parietal cells and other cells of the gastric mucosa Only parietal cell cAMP formation is stimulated by histamine

METHODS

Isolation of cells from rat mucosa

A method modified from Lewin et al (10) was used After killing 15 rats by a blow on the head the stomachs were removed Antra and rumina were removed The stomach remnants were transformed into everted sacs These sacs were filled with 1000 PUK/ml pronase solution and incubated for 90 minutes in a gassed (95% O_2/5% CO_2) Ca^{++} free Hepes/bicarbonate buffer solution The everted sacs were then gently stirred in a Hepes buffer solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 minutes Four fractions of isolated cells were obtained and filtered through a nylon mesh Finally the cells were washed twice to remove all pronase and resuspended to give a final concentration of 3-5 x 10^7 cells/ml

In order to obtain fractions enriched in parietal cells a serial centrifugation technique modified from Lewin et al (10) was used (fig 1)

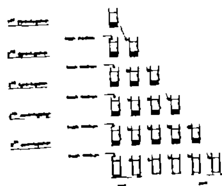


Fig 1 Serial centrifugation of isolated gastric cells
All centrifugation steps are carried out at 100 g for 45 seconds Each pellet remains in the centrifugation tube and is resuspended by the supernatant from the tube on its left The pellet in the first tube is resuspended by fresh medium; the supernatant of the tube to far right is transferred into an empty tube After 5 centrifugation steps pool I contains 45% and pool II ^%

Measurement of cAMP formation

A 0 5 ml sample of isolated cells was added to 5 ml of the gassed incubation mixture containing Hepes-buffer at pH 7 4 The mixture was gently shaken in a water bath at 37^{O} C At zero time the compound(s) to be tested were added together with 10^{-2}M theophylline After different time periods 0 5 ml samples were transferred from the incubation mixture into tubes which were kept in liquid nitrogen cAMP was extracted by trichloracetate and acetylated according to the method by Fraudsen and Krishna (7) A radioimmunoassay with antisera from Schwartz and Man was performed

Assessment of cell viability

The cells were counted in a Fuchs-Rosental chamber The rate of cell damage was evaluated by a trypan blue exclusion method Within 60 minutes the number of unstained cells dropped from initially 93 7 ± 6 3% to 89 2 ± 8 8 % (mean ± SD) Pools in which the number of unstained cells was lower than 85 % at the beginning of the experiment were discarded In previous experiments the cells of this type of preparation have been shown to be viable on the basis of their ability to maintain transmembrane ion gradients (H R Koelz Amer J Physiol in print)

Identification of parietal cells

Parietal cells were identified by staining their succinylic dehydrogenase content with nitro-blue tetrazolium (3)

RESULTS

The cAMP content of unstimulated cells was 0 45 ± 0 17 pmoles/10^6 cells (mean ± SD) and remained constant during the 65 minutes observation period By adding theophylline at a concentration of 10^{-2}M the cAMP content more than doubled within 15 minutes and remained constant thereafter These results were independent of the parietal cell content (fig 2)

The histamine-1 receptor antagonists diphenhydramine and mepyramine also inhibited the effect of histamine but only at concentrations 100 to 1000 times higher than the lowest effective dose of cimetidine (fig 6)

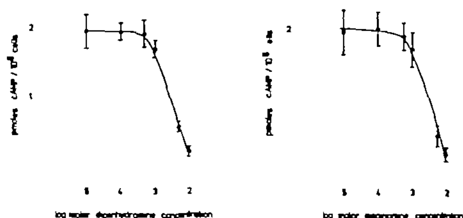


Fig 6 Effect of diphenhydramine and mepyramine on cAMP formation in the presence of 10^{-4}M histamine and 10^{-2}M theophylline ($\bar{X} \pm$ SEM n = 4)

At a concentration of 10^{-2}M the two histamine-1 receptor antagonists reduced cAMP concentration below theophylline levels This effect was observed with parietal cell concentration ranging between 10 and 50% Figure 7 shows the effect of prostaglandin E_1 at a concentration of 1 µg/ml on pools with a low parietal cell content When in addition to theophylline

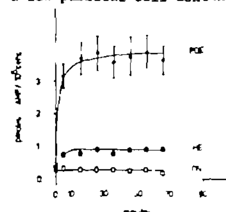


Fig 7 cAMP concentration after stimulation by prostaglandin E_1 PGE_1: 1 µg/ml prostaglandin E_1 given together with 10^{-2}M theophylline THE: 10^{-2}M theophylline given alone CON: unstimulated controls Each point is the mean $\pm$ SEM of 12 independent measurements Differences between prostaglandin stimulation theophylline and control values are statistically significant $p < 0.001$

prostaglandin E_1 was given a further fourfold increase of cAMP content was observed Cimetidine and propranolol had no effect on prostaglandin E_1 stimulation of cAMP formation The parietal cell content of each pool was compared to the cAMP concentration stimulated by prostaglandin E_1 A negative linear regression was obtained (fig 8)

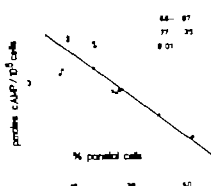


Fig 8
Relationship between prostaglandin E_1 stimulated cAMP concentration and parietal cell content in per cent of each fraction Each point is the mean of 5 consecutive measurements in the same incubation mixture

DISCUSSION

In the present study the effect of histamine and prostaglandin E_1 on i olated viable rat gastric cells was studied By a simple centrifugation technique cell pools with different parietal cell contents were obtained without effecting cell viability In all tests theophylline was given as a background stimulation in order to produce high and long lasting elevation of cAMP Interestingly the effect of theophylline was similar in pools with a high and low parietal cell content In all in tances theophylline doubled cAMP content

The stimulation of cAMP formation in the gastric mucosa by histamine and prostaglandin (2 6 12 17 20) was confirmed in the present study However the two compounds appear to act on two different types of cells Therefore previous studies in which whole gastric mucosa has been examined may give misleading information on the mechanism by which histamine and prostaglandin effect gastric secretion (12 18 19 20)

The response of a cell pool to histamine wa linearly dependent on its parietal cell content From linear extrapolation of a regression analysis (fig 3) it is possible to calculate the effect of histamine on pure parietal cells (3 46 ±

1 79 pmoles cAMP/10^6 cells) and on parietal cell free pools (0 89 $\pm$ 0 46 pmoles cAMP/10^6 cells) The latter value is nearly identical to the cAMP content of unstimulated mucosal cells Therefore histamine appears to exert its effect only on parietal cells Here histamine appears to act on H_2-receptors since its effect is completely inhibited by cimetidine a specific histamine-2 blocking agent Histamine stimulated cAMP was also inhibited by diphenhydramine and mepyramine two histamine-1 receptor blocking agents This effect however occured only at extremely high concentrations which also reduced theophylline stimulated cAMP content in the absence of histamine Therefore the effect of histamin-1 receptor antagonists appears to be nonspecific An unlikely explanation of the effects observed with histamine-1-receptors blocking agents is the presence within the suspension of mast cells which released effective amounts of endogenous histamine in the absence of exogenous histamine The volume of the incubation mixture is 100 to 1000 times higher than the volume of the intact tissue Therefore the concentration of endogenous histamine would be diluted by at least this factor Furthermore the histamine-1 blockers reduce the cAMP content even below the basal level of the tissue

Prostaglandin E_1 in contrast to histamine appears to raise cAMP of non-parietal cells Within the range of the parietal cell contents obtained by our centrifugation method the relationship between this prostaglandin E_1 effect and the parietal cell content is linear By linear extrapolation the effect of prostaglandin on pools with a parietal cell content of more than 60% can be calculated Negative values are obtained for pools with a parietal cell content of more than 70% The most likely explanation for this finding is an inhibition of cAMP formation in parietal cells An alternative explanation is a nonlinear relationship between parietal cell content and cAMP formation

It has been postulated that cAMP is the second messenger of gastric acid secretion stimulated by histamine (2 4 13 16) but this role is controversial (1 8 18 19) The data of the present study are compatible with a second messenger role of cAMP because histamine which stimulates gastric acid secretion stimulates the cAMP content of the parietal cells and prostaglandin E_1 which inhibits acid secretion (11 14 15) has no effect on or may even inhibit cAMP formation by these cells The physiological significance of cAMP formation stimulated by prostaglandin E_1 in nonparietal cells is at present unknown

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Active Transport of Sodium, Respiration and Phosphate Metabolism in the Foetal Gastric Mucosa

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ABSTRACT: Changes in active transport of Na J_{Na} by rabbit foetal gastric mucosa do not follow changes in ATP or ATP/ADP during recovery from anoxia or ouabain inhibition During anoxia phosphorylcreatine PCr and ATP levels do decline in parallel with J_{Na} When Na dependent respiration as well as J_{Na} ATP/ADP and PCr are all measured the observed interrelationships can be accounted for if a high energy state (electrochemical activity) common to electron transport and phosphorylation of ADP is the immediate energy source for J_{Na}

There is now a considerable body of evidence to substantiate the theory that the plasma membrane Na^+ pump is energised by the phosphoryl group transfer potential available from the hydrolysis of ATP; the energy being coupled to Na^+/K^+ transport through an enzyme which is an integral component of the membrane structure (1) Such evidence has been obtained almost entirely under conditions in which ATP is not being produced by oxidative phosphorylation as in red cells and ghosts (2-4) or where oxidative phosphorylation has been blocked (5) More recent work has shown that the purified $(Na^+ + K^+)$-transport ATPase can be incorporated into the lipid membrane of artificial vesicles when all the standard properties of the transport system are exhibited Mg^{2+}-ATP-dependence ouabain inhibition asymmetry etc (6)

Studies of active transport of Na^+ across epithelia under aerobic conditions have shown a stoichiometry between rate of transport and oxygen utilisation (7-9) Since the observed active transport has been shown to be inhibited by cardiac

glycosides (10 11) it is generally presumed that the final mediator of the transport is the $(Na^+ + K^+)$-ATPase system; and therefore there is only an indirect dependence of active transport on respiration

The present paper reports an attempt to measure the interdependence of respiration phosphate metabolism and rate of active Na^+ transport; and thus to test the above presumption The preparation chosen was the 28 day rabbit foetal gastric mucosa: this is a mammalian epithelial Na^+ transport system which is viable for several hours and whose basic transport properties have been demonstrated previously (12 13)

METHODS

The rate of active Na^+ transport was measured as short-circuit current (s c c) in the presence and absence of Na^+ on the mucosal side (13) The applicability of the method under extreme conditions e g anoxia was confirmed (Rice-Evans & Wright unpublished) Mucosal cell suspensions were incubated at 35°C in Krebs bicarbonate buffered solution (as used in the s c c experiments see (13)) gassed with 95% O_2 + 5% CO_2 or 95% N_2 + 5% CO_2 in anoxic experiments In Na^+ free experiments the Na^+ was replaced by an equivalent amount of choline

Oxygen consumption was measured on cell suspensions using a Clarke-type oxygen electrode For extraction and analysis of ATP ADP and phosphorylcreatine PCr using the method of Lowry et al (14) cell suspensions were centrifuged at 1700 x g for 10 min at 1°C; the cell pellet obtained was rapidly weighed and frozen in liquid N_2

RESULTS

Viability of the cell suspensions A value of Q_{O_2} of 0 27 ± 0 07 (n = 5) μ-moles O_2/mg dry wt /hr obtained under control conditions in the presence of Na^+ agrees closely with values reported by other authors working on suspensions of cell of transport epithelia e g (15) In Na^+-free Krebs solu c Q_{O_2} was 0 17 ± 0 04 (n = 4) μ-moles O_2/mg dry wt /h demonstrating that over 50% of the respiration was Na^+ depe d t and hence performed by an intact cellular

system

Concentrations of ATP ADP and PCr (μ-moles/g cell water) under control conditions were 0 19 ± 0 03 0 35 ± 0 04 and 1 74 ± 0 31 (n = 6) respectively These values may be compared with those obtained by Durbin & Michelangeli (16) from isolated frog gastric mucosa after recalculating their figures using a value of 23% for the extracellular water of this tissue (17) to obtain the following concentrations (μ-moles/g cell water): 1 26 ± 0 08 0 19 ± 0 01 and 1 17 ± 0 06 for ATP ADP and PCr respectively

It should be noted that in the foetal cells the concentration of the highly labile PCr is an order of magnitude greater than that of the relatively stable ATP this situation would only persist in intact viable cells The ratio ATP/ADP at about 0 5 is an order of magnitude lower than that obtained for frog gastric mucosa; but there is no reason a priori why this should not be so It should be noted that ATP + PCr concentrations are similar in both the foetal gastric cells and the frog gastric mucosa Examination of the cells by conventional light microscopy after incubation for 4 hr and application of the eosin exclusion test provided no evidence of cell damage It is concluded that the foregoing criteria provide adequate evidence of the viability of the cell suspensions used in this work

The effect of anoxia When the preparations were rapidly made anoxic (Fig 1) the decline in total s c c and Na^+ dependent s c c (J_{Na}) followed approximately a single exponential The total s c c and J_{Na} fell to 6% and 9% respectively of the initial values in 1 hr There was a large decrease in the ATP and PCr concentrations in the presence of Na^+ both along a time course similar to the fall in s c c At 1 hr the ATP concentration was very low and there was a considerable fall in the value of ATP/ADP to about 0 2 There was no significant further decline in PCr after 1 hr which remained at about 50% of the pre anoxic level In the absence of Na^+ delayed decreases in PCr and ATP concentrations and ATP/ADP were seen



Fig 1 The effect of anoxia from time O
● —— ● + Na^+
o --- o − Na^+
Each point represents the mean ± S E of mean (n = 5)

The effect of reoxygenation after 1 hour of anoxia In Fig 2 it is seen that upon reoxygenation the Na^+ dependent Q_{O_2} very rapidly returned to about 90% of the pre-anoxic rate and then fell in a linear manner to a minimum of about 55% of the pre-anoxic rate at about 1 25 hrs after which it rose again at a rate comparable to the fall Over the same period J_{Na} increased initially at a maximum rate reaching a maximum of 82% of the pre-anoxic value at about 1 hr after which a slow decline occurred There was no recovery of the Na^+ independent s c c

On reoxygenation a rapid rise in the ATP concentration and ATP/ADP was seen After an initial delay of about 15 min the PCr concentration declined for about 45 min at a similar rate to that seen during the first hour of anoxia after which it

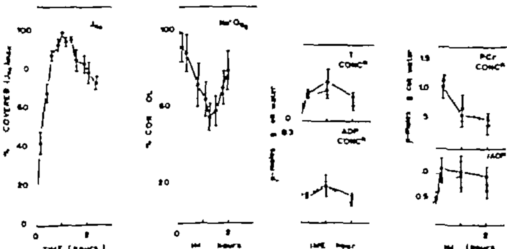


Fig 2 The effect of reoxygenation (from time O) after 1 hr of anoxia ● —— ● + Na^+ o --- o − Na^+ Each point represents the mean ± S E of mean (n = 5)

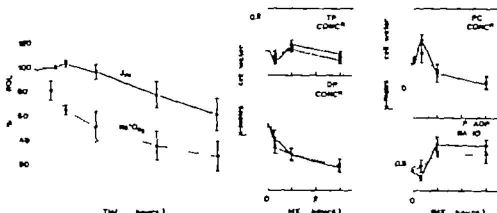


Fig 3 The effect of ouabain (10^{-5}M) from time 0 on J_{Na} and $Na^{+}Q_{O_2}$ each point represents the mean ± S E of mean (n = 4); and on ATP ADP and PCr concentrations and ATP/ADP ● —— ● + Na^{+}; o —— o - Na^{+} each point represents the mean ± S E of mean (n = 6)

plateaued out

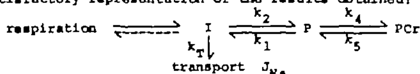
The effect of ouabain Fig 3 shows that the effect of 10^{-5}M ouabain on Na^{+} transport and metabolism could be divided into two phases The first phase occurred during the first hour during which J_{Na} remained steady and the Na^{+} dependent Q_{O_2} declined at a maximum rate There was a transient decline in the ATP concentration and ATP/ADP in the presence of Na^{+} and a transient increase in the PCr concentration The second phase began after 1 hr when a steady and parallel decline in J_{Na} and Na^{+} dependent Q_{O_2} was observed over the following 2 hrs During this phase there was no significant change in the ATP and PCr concentrations; ATP/ADP was raised in the presence of Na^{+} to about unity double its initial value

DISCUSSION

If hydrolysis of ATP is the only mechanism for directly supplying energy to the transport mechanism there should be a consistent relationship between the cell ATP concentration or more precisely ATP/ADP (as this is an indicator of phosphoryl group transfer potential) and the rate of Na^{+} transport During the rapid decline in Na^{+} transport with the imposition of anoxia there was a decline in ATP/ADP to a very low value followed by a slow increase which was not associa-

ted with recovery of Na^+ transport; this could be due to the very low cell ATP concentration persisting Results of this type have been reported for frog gastric mucosa in relation to transport of H^+ and Cl^- and considered to verify the primary role of ATP hydrolysis in the active transport of ions (16 18) On recovery from anoxia ATP/ADP rose rapidly and levelled out at the relatively high value of 1 0 at 1 hr; in spite of this Na^+ transport at this time started to decline having reached its maximum

The results presented here require a careful examination of the relationship between the ($Na^+ + K^+$)-transport ATPase system and oxidative phosphorylation with particular regard to the presumption that active transport is under all conditions energised directly by ATP Since the predicted relationship between Na^+ transport and ATP concentration or ATP/ADP was not invariably obeyed the following scheme may provide a satisfactory representation of the results obtained:



In this model the immediate energy source for active transport is the intermediate I the rate of Na^+ transport J_{Na} being given by $J_{Na} = k_T I$ where k_T is a rate constant The dashed arrow represents a feed-back providing respiratory control P represents the ATP activity at the pump site and will also be taken as synonomous with ATP/ADP PCr is the phosphorylcreatine activity in the region of the pump site The subscripted k s are rate constants Na^+ dependent respiration is an empirical function of time and will be written R(t) The kinetic equations for the model are then as follows:

$$\frac{d[I]}{dt} = R(t) + k_1[P] - k_2[I] - k_T[I] \quad (1)$$

$$\frac{d[P]}{dt} = k_2[I] - k_1[P] + k_5[PCr] - k_4[P] \quad (2)$$

From (2) $k_1[P] - k_2[I] = k_5[PCr] - k_4[P] - \frac{d[P]}{dt}$ (3)

Substituting (3) into (1) and noting that

$$k_5[PCr] - k_4[P] = -\frac{d[PCr]}{dt}$$

$$\frac{d[I]}{dt} = R(t) - \frac{d[PCr]}{dt} - \frac{d[P]}{dt} - k_T[I] \tag{4}$$

Since $\frac{d[P]}{dt} \ll \frac{d[PCr]}{dt}$ (4) becomes after rearrangement

$$\frac{d[I]}{dt} + k_T[I] = R(t) - \frac{d[PCr]}{dt} \tag{5}$$

As $k_T[I] = J_{Na}$

$$\frac{1}{k_T}\frac{dJ_{Na}}{dt} + J_{Na} = R(t) - \frac{d[PCr]}{dt} \tag{6}$$

Equation (6) indicates that in general the rate of active transport of Na^+ is determined by the rate constant k_T the Na^+ dependent oxygen consumption R(t) and the overall rate of utilisation of ATP measured in this particular system as the rate of decline in PCr The applicability of equation (6) under various experimental conditions has been tested; in particular to the recovery from anoxia the effects of anoxia, and the effects of ouabain

It should be noted that the kinetic model excludes significant Na^+ dependent production of ATP by anaerobic glycolysis. Control experiments (unpublished) have shown that under the conditions in which these experiments were performed there was no Na^+ dependent production of lactate

In Fig 4 the computed recovery of J_{Na} during reoxygenation after 1 hr of anoxia is shown along with the Na^+ dependent respiration and PCr utilisation the negative differential of PCr in the presence of Na^+ determined from the results shown in Fig 2 The agreement between the observed and computed recovery is satisfactory when $k_T = 2\ 6\ hr^{-1}$ Although it may be argued that this could arise because of a lack of restriction on the value of k_T an estimate of the correct value of this parameter may be obtained explicitly if it is noted that during the first part of recovery R(t) and $-d[PCr]/dt$ are approximately equal and opposite so that equation (6) becomes

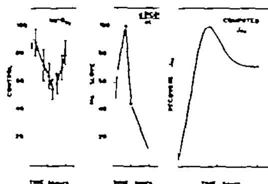


Fig 4 The effect of reoxygenation after 1 hr of anoxia For $Na^+Q_{O_2}$ each point represents the mean ± S E of mean (n = 5)

$$\frac{dJ_{Na}}{dt} + k_T J_{Na} = \text{constant}$$

The solution of this equation provides an estimate of k_T from the slope of $\ln\left(\frac{100 - J_{Na}}{100}\right)$ plotted against time J_{Na} being expressed as a percentage of the maximum transport rate recovered Fig 5 shows the semi-log plot obtained The slope of the regression line gives $k_T = 2\ 8\ hr^{-1}$ during the first part of recovery (correlation coefficient = 0 8993 p<0 001)

It is also possible to predict the changes in Na^+ transport with the onset of anoxia (R(t) = 0) Thus under this condition the differential equation (6) takes the form in which its solution must be of the same form as $\frac{d[PCr]}{dt}$

The results given in Fig 1 enable $-\frac{d[PCr]}{dt}$ to be determined: a semi-log plot showed this function to be given by:

$$\left(\frac{d[PCr]}{dt}\right)_t = \left(\frac{d[PCr]}{dt}\right)_{t\,=\,0} e^{-3\ 33t} \qquad (7)$$

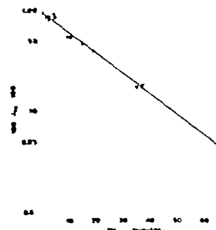
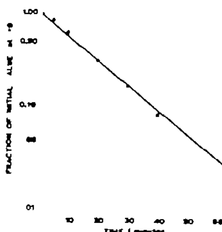


Fig 5 Initial recovery of J_{Na} on reoxygenation after 1 hr of anoxia J_{Na} is expressed as % of maximum recovered (at 1 hr) $\frac{dJ_{Na}}{dt} + k_T J_{Na} = 100$ Each set of symbols represents the data of a single experiment

Fig 6 J_{Na} (● —— ●) and PCr utilisation $d[PCr]/dt$ (o —— o) during anoxia The slope of the line corresponds to a rate constant = 3 33 hr^{-1} (correlation coefficient = 0 9923 p<0 001) The data is derived from Fig 1



The solution of the general equation for J_{Na} (equation (6)) should then be of the same form as equation (7) with the same rate constant Fig 6 shows that during anoxia J_{Na} and $\frac{d[PCr]}{dt}_t$ follow the time course determined by the latter

The general equation (6) should describe the effects of ouabain if this agent is acting directly on the pump mechanism i e reducing k_T as generally accepted and not as a metabolic inhibitor i e k_1 k_2 k_4 and k_5 are unaffected The equation thus becomes nonlinear and problems of its solution arise In a subsequent paper it will be shown by analogue computation based on the kinetic model presented in this paper and without recourse to the empirical functions R(t) and $-\frac{d[PCr]}{dt}$ that the effect of ouabain is simulated quite well if k_T suffers an exponential decline with a rate constant of 1 0 hr^{-1} The results presented here show that 1 hr after ouabain administration ATP and PCr concentrations remained constant and J_{Na} appeared to be related to R(t) alone

If the results presented here can be explained by the immediate energy source for active transport being an electrochemical activity common to energy pressure from respiration on the one hand and from the ATP-ADP couple on the other it is necessary for any proposed mechanism to involve a high-energy state common to the general transport ATPase (common to aerobic and anaerobic systems) and the high-energy intermediate of oxidative phosphorylation A possible mechanism

is of the type proposed by Mitchell (19 20) for the mitochondrial respiration dependent and ATPase linked proton pump adapted to its more general form as a cation pump realising that the intermediate electrochemical activity could be simply a membrane potential or a chemical entity or a combination of the two (19)

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Is Anion-sensitive ATPase a Plasma Membrane Located Transport System?

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ABSTRACT: Studie on the alleged role f anion-sensitive Mg-ATPas in anion and proton transport ros the plasma membrane ar reported It is concluded that the enzyme is located in mitochondria b t not in the plasma membrane in rabbit gastric muco trout gill r bbit kidney and r t pancreas wheras in r bbit rythrocyte membrane it is part f the Ca-Mg activ ted ATPase ystem These findings appear to rul out function f the anion-sensitive ATP s in th transport f anions and protons cros the plasma membrane f these tissues

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Introduction

It has been suggested i 1965 by Durbi nd Kasbekar (1) that anion-sensitive Mg-ATPas and carboni anhyd s ar involved in the ga tric acid ecr tion p oce Thi idea was trenghtened by the bserv tion that the ATPas i stimulated by bicarbonate and that both the ATPas nd id secr tion r inhibited by thiocyanat In addition the r latively high carbonic anhyd as activity in th ga tri mucosa (2) and the inhibition f ga tric id secr tion by the carboni anhydrase inhibit r ac tazol amid sugge ted that this enzyme could l be involved in the secr t ry proc

Th model p opo ed by Durbin and Kasbekar (1) w based on the mechanism f the Na-K ATPase cation transport y tem Bicarbonate and chlorid t gethe would stimul te the enzyme leadi g to phosphorylation by ATP and t imwa d bicarbonat and twa d chloride transport Bi rbonat plus proton would res lt f om the hyd tion f CO_2 by the carbonic nhydras The pr ton and hlorid ion would onstitut HCl ecr tion

Since the many eport f tudi of the nion- ensitive Mg-ATP activity ga tri muco and the ti ues have ppe ed The p enc

f both a mitochondrial and a non-mitochondrial activity in gastric mucosa was claimed (3 4) Unfortunately the properties of these two activities strongly resembled each other (5) On the other hand Soumarmon et al (6) could only detect a mitochondrial but not a plasma membrane bound anion-sensitive Mg-ATPase activity in rat fundus mucosa Since mitochondria are abundant in the parietal cell (7) contamination of a so-called plasma membrane fraction with mitochondrial fragments cannot easily be prevented

Nature f the anion effect

The anion-sensitive ATPase is stimulated not only by HCO_3^- but also by various other oxyanions like borate lenite arsenite ars nat and sulfite (3 4)

In a study of the enzyme activity in lizard gastric mucosa using media with only one anion present which is gradually being replaced by anoth r anion (8) we have observed that the enzyme appears to have only one anionic binding ite rather than two as suggested by Durbin and Kasbekar (1) We also found that the enzyme activity i inversely proportional to

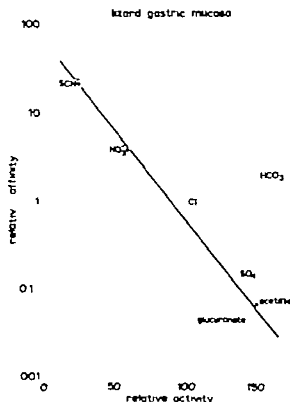


Fig 1

Relationship between r lative anion affinity of Mg^{2} -ATPa and the relative enzyme activity in the pr sence of each anion (from ref 8)

the logarithm of th affinity f the anion to th enzyme (fig 1) although bicarbonate gives bout twice the activity expected Thus the enzyme activity actually seems to be inhibited when an anion i bound to the anionic binding sit

Thi ituation differs sharply from that in Na-K ATPase which has two cationi binding ites one for Na and one f r K^+ and where activation of the enzyme takes pla upon binding of the two cations Finally the mola rati f H^+ transported and ATP hydrolysed by the anion-sensitive ATPase was very low (0 06–0 17) compared to the Na^+/ATP rati f 3 for Na-K ATPase

The findings ggested that the mod 1 of Durbin and Kasbekar (1) would t least need modification More s rious even was th unc rtainty abo t a localization f the anion-sensitive ATP in the plasma membrane Such localization i f cour cruci l for its alleged role in gastri acid secretion and th r f rms of active anion transport acro the plasma membrane Hence we have med a critical tudy f the intracellular origin and chara teristics f th enzyme in g stric mucosa and thar ti sues in which th enzyme had been reported (gill kidney pancreas erythrocyt)

ATPas activiti in the main fra tions f gastri mucos

The fractionation techniques have been described lsewhe e (9 10) Fig 2 hows the distribution f Mg-ATP ctivity in the main fra tions of rabbit gastri mucosa Th large t timulatory effect f HCO_3^- and inhibitory effect of SCN i b erved in the heavy mitochondrial (10 min

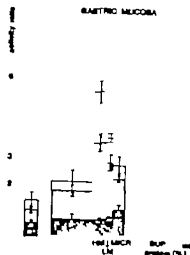


Fig 2

Distribution f anion-sensitive ATPa a tiviti in main fra tions of gastri mucosa Ratios f the specific activity in HCO_3 medium (t tal column) Cl medium (haded part f column) and SCN^- medium (darke t part f column only) to the specifi ctivity f th homogenate in Cl medium are pl tted gainst the p ot in distribution in percent Abbreviation H i the homogenat N HM LM and MICR ar th fractions sedimenting at 10 min 1 000 x g 10 min 10 000 x g 20 min 20 000 x g and 60 min 100 000 x g espectively SUP is the remaining upernatant Means of five ex periments with tandard rrors a given (from f 9)

10 000 x g) fraction but the light mitochondrial (20 min 20 000 x g) and microsomal (60 min 100 000 x g) fractions r also anion-sensitive The Mg-ATPase activity in the supernatant is negligible which indicates that the enzyme activity is membrane bound

Most authors who claim to have found a non-mitochondrial anion-sensitive Mg-ATPase used a micro omal fraction Henc we have subjected the microsomal (60 min 100 000 x g) fraction to sucros density gradient centrifugation and have determined the distribution patt rn of th enzyme in the subfractions thus obtained

ATPase activities in the microsomal fr tion of gastric mucosa

The distribution pattern of the microsomal (60 min 100 000 x g) fraction is shown in fig 3 In thi fraction two HCO_3^--stimulated Mg-ATPas peaks re observed one sedimenting at 40–44% (w/v) sucrose (peak II) and one sedimenting at 48–52% (w/v) sucrose (peak III) Peak III coincides with the highest lev l of cytochrome c oxidas ctivity and thus appears to be of mitochondrial origin The Mg-ATPas ctivity at the top of the gradient (peak I) coincides with the highest level of 5 nucleotidase

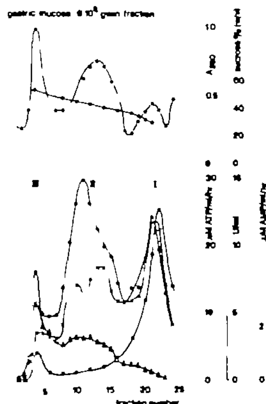


Fig 3

Enzyme distribution pattern after density gradi nt centrifugation (16 h 64 000 x g) of microsomal (60 min 100 000 x g) fraction of rabbit gastri muc sa (typical experiment representative fo 28 experiments)
Symbol : top: -•- distribution of sucrose; - 280 nm ab orbance after dilution and correction; bottom: -o- ATPase tivity in HCO_3^- medium; -x- ATPase cti ity in Cl^- medium; -Δ cytochrome c oxidas activity; -□- 5 nucleotidase a tivity
(from r f 9)

activity and is with the K^+-stimul ted pho phatase nd (Na-K)ATP e activities and thus this peak probably repr ent plasma memb ane fraction Thi fraction is el tively insensitive to stimul tion by HCO_3^- and t inhibition by thiocyanate A plasma memb ane f ction lacking anion-sensitive ATPase activity was ls btained in gastric mucosal micro ome f pig by F rt t al (11) and f r t by Soumarmon et al (6)

The low cytochrome oxidas lev l in peak II could uggest a non-mitochondrial origin f thi fraction However the intracellul r markers like glucose-6-pho phatas (smooth endoplasmic r ticulum) RNA (rough endoplasmic reticulum) and (Na-K)ATPas (plasma memb ne) do not coincid with the HCO_3-stimul ted ATPase a tivity in peak II Monoamine oxidas marker enzyme f r the mitochondrial outer membran (12) has a di tribution which partially par llel that f the cytochrome oxidas activity but it is also found at the top of the gradient where an anion-insensitive Mg-ATPas (peak I) is p sent Thi gge ts that the mitochondrial anion-ensitive Mg-ATP se activity is not located in the mitochondri l out r membran

Nature f peak II anion- ensitive Mg-ATP

Since th peak II anion-sensitive Mg-ATPas a tivity does not coincide with various marker enzyme activities f plasma membran endoplasmi reticulum, but only with low level f cytochrome oxidas or monoamin oxidase activity we have furth r tudied the natur f thi ATP by comparing it chara t ristics with tho f th peak III anion-sensitive Mg-ATPas tivity which ppears to be f mitochondrial rigin The ef f cts f various inhibit ry sub tances on th anion- ensitive Mg-ATPase activity in the two subf actions f th gastri mu sal microsomes summarized in tabl 1 The pI_{50}-value f the two prepar tions and lso the rest activiti emaining t maximal inhibition re virtually identi cal The inhibition f th two tivities by aurovertin and ligomycin and particularly th nea ly compl t inhibition by combination of thes two sub tance trongly sugge ts mitochondri l rigin for both tivi ti

Further evidenc i obtained from the effects f abbit live mitochondrial inhibit prot in (13) on both peaks The results in fig 4 how that the mitochondrial inhibito protein affect both anion-sensitive Mg-ATP se activity peaks At inhibit /ATP se-rati of 205 and 369 µg inhibit unit [1] ATPas espectively 50% inhibition of ATPas activity i

10 000 x g) fraction but the light mitochondri 1 (20 min 20 000 x g) and microsomal (60 min 100 000 x g) fractions are also anion-sensitive The Mg-ATPase activity in the supernatant i negligible which indicat that th enzyme activity is membran bound

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ATPase activities in the microsomal fraction of gastric mucosa

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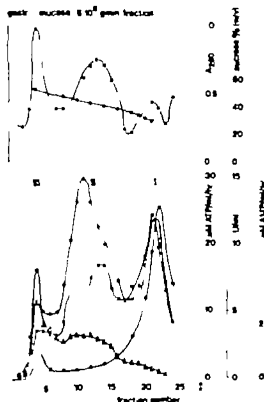


Fig 3

Enzyme distribution pattern after density gradient centrifugation (16 h 64 000 x g) of microsomal (60 min 100 000 x g) fraction of rabbit gastric mucosa (typical experiment repres ntative for 28 experiment)
Symbols: top: -e distribution of sucro ; * 280 nm bs bance ft r dil tion and c rrection; bottom: -o- ATP se activity in HCO_3 medium; -x- ATPase ctivity in Cl^- medium; -Δ cytochrome oxidas activity; -□- 5 nucleotida activity
(from ref 9)

obtained Thi indicates a somewhat higher ensitivity f the (peak II) ATPase activity which may reflect that these submitochondri l parti l s are more inhibitor-depl ted

The phospholipid omposition f both peaks t g ther with that f subfraction I is pre ented in table 2 Th r is a lose re emblance between the pho pholipid patt rns of peak II and III Peak I shows lower level f phosphatidyl holine and phosphatidylinositol and higher levels of phosphatidylserine and sphingomyelin which is more lik th phospholipid composition f plasma memb anes Th high l vel f diphosphatidylglyc rol (cardi lipin) in peak II ugge ts that thi fra tion is enriched in mitochondrial inner memb ane (14 15)

The detergent Triton X 100 solubilizes the enzyme activity With increa ing Triton X 100/prot in ratios the pecific activity f anion-ensitive Mg-ATPas in the supernatant of the microsomal fra tion f rabbit gastri mucos increases whil that remaining in th pellet decreas (fig 5) The ptimal r ti i eached at a d tergent/protein ratio f 3:1 The amount f protein which i s lubilized increases sharply up to a atio f 1:1 and then rises slowly toward higher ratios

Since littl enzyme a tivity i solubili ed t ratio f 1:1 thi ff the pos ibility of purifying the enzyme tivity by means f tep-wis lubili tion Th fractions btained from gastri mucosal microsomes by means of such step-wise solubilization procedure have been analysed by SDS-g l lectrophor i We ob erve two dominant protein bands with molecular weights f 64 200 Dalton and 61 600 Dalton and lesser

TABLE 2

PHOSPHOLIPID COMPOSITION OF THREE SUBFRACTIONS OF RABBIT GASTRIC MUCOSA

Pho pholipid	Subfra tion I	II	III
Phosphatidyl thanolamin	28 0 ± 1 0	28 6 ± 0 5	24 4 ± 0 7
Phosphatidylcholine	27 2 ± 0 7	43 5 ± 1 5	49 6 ± 0 7
Phosphatidylinositol	4 7 ± 0 3	6 0 ± 0 4	6 6 ± 0 3
Phosphatidylserine	13 1 ± 1 0	5 3 ± 0 4	4 2 ± 0 2
Sphingomyelin	18 8 ± 1 5	6 7 ± 1 0	6 2 ± 0 7
Lysopho phatidylcholine	2 5 ± 1 6	–	0 9 ± 0 4
Diphosphatidylgly er l (cardi lipin)	1 2 ± 0 4	7 0 ± 1 1	3 1 ± 0 5
Unidentified	4 7 ± 0 6	2 7 ± 0 8	4 2 ± 1 0

Microsomal fraction of rabbit gastri mucosa ubjected t density gradient entrifug tion V lue p rcentages f total phosphoru with tandard rror f four exp riments

band of 28 500 Dalton (means of two experiments) This protein distribution is very similar to the subunit p tt rn of mitochondrial F_1 ATPase from various tissues (16) but differs from the subunit composition of (Na-K)ATPa e and Ca-Mg-ATPase which both contain a subunit of about 100 000 Dalton and a subunit of lower molecular weight

Electron micro copic observations on subfractions of g stric mu osa

Peak I consists mainly of ve icular structures and could originate from the pl sma membrane or from microvilli located on the apical cell surfac of the pari tal cell The ATPase activity in this subfraction is not anion-sensitive in ontrast to that in the morphologically similar vesicular fraction btained t slightly high r densities (1 09 1 12) from necturus oxyntic c 11 and dog gastric mucosa by Wiebelhaus et al (17) and Blum et al (3) Peak III contains rough endoplasmic reticulum and a considerable number of small si ed mitochondria Peak II has fewer mitochondria most of them l cking an out r membrane whil lysosomes rough and possibly smooth endoplasmic reticulum re also present in this fraction

F rther experiments on the origin f peak II

The results f th inhibitor studies the phospholipid compo ition and the gel lectrophoretic studies trongly uggest that pe k II which contains the major anion-sensitive ATPas activity in th microsomal f action is like peak III of mitochondrial origin

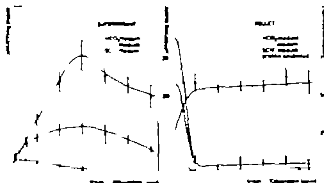


Fig 5
Effect f T iton X-100 on the solubility of anion- ensitive ATPase activity in th microsomal fraction of rabbit g stri mucos Me n of 3 experiments ar shown with the tandard rror Symbol : -o- ATPase activity in HCO_3 medium; -•- ATP a tivity in Cl^- medium; Δ- ATPa ctivity in SCN^- medium; -□- p rcent g f protein solubilized

The diff rence in density of peak II and peak III and the different r tios between cytochrome c oxidase and anion- ensitive ATPase activity in these peaks still requir an explanation

The higher anion-sensitive Mg-ATPase to cytochrome c oxida ratio in peak II compared to peak III could r flect activ tion through loss f an ATPase inhibitor prot in (18) or an increase in sub trat acce ibility f the ATPase during purification

Recentrifugation of peaks II and III pooled after an initial epara tion by the normal procedure for 16 h in a density gradient results in the return of both peaks t the ame places in th gradient which they occupied after th initi l centrifugation Thi indicate that the peak II mat rial is not formed from peak III during the initial entrif gation When the fractions (microsome 60 min 100 000 x g and light mitochondria 20 min 20 000 x g) resuspended in 55% (w/v) sucro re placed on top of layer f 64 5% (w/v) sucros and ubjected to centrifugation by fl ta- tion in gradient of 55 to 30% (w/v) sucrose peak ctivitie of nion- sensitive Mg-ATPa ar lso found at the normal places in the gradient

Prolonged centrif g tion f microsomal and mitochondri l fractions on a normal ucro e gradient for 65 h inste d f the usual 16 h yi lds about the same enzyme distribution pattern indicating that aft r 16 h equi- librium has been reached However when gradient centrif gation is carried out for only 8 h diff rent pattern i obtained (fig 6) At that time peak fra tion III has lready reached its normal po ition indicating that

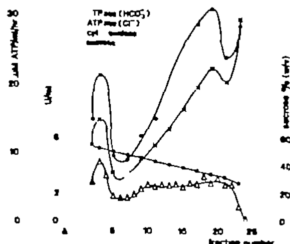


Fig 6
Enzyme di tribution f gastric mucosal microsomes on the normal density gradient after 8 h instead f th su- l 16–17 h centrifugation t 64 000 x g_{av} Activiti of ATPas in HCO_3^- medium ( ) nd Cl medium (x) tog ther with cytochrome oxidas (Δ) and ucros concent tion ( ) re hown Typical experiment re- pre entati f two experi ments
(from ref 9)

it consists of rather large particl Peak fraction II is then still near the top of the gradient indicating that the particles in this fraction which contain the peak II HCO_3^- ATPase activity must be smaller than those in fraction III They could originate from disruption of mitochondria which are bundant in the peri tal cell of th gastric mucosa (7) This would also gree with the mor evenly distributed cytochrome c oxida e ctivity in this case

It thus appears that drastic homogenization of the gastric mucosa could disrupt the mitochondria to such an extent that ft r 16 h of centrif ga-tion peak II has not yet reached equilibrium This may pos ibly expl in why Sachs et al (4) appear to find an anion- ensitive ATPa e activity of gastric mucosa in an apparent plasma membrane f action in the lower-density region of their gradients Unfortunately they did not d scribe their tissue preparation and homogenization methods in d tail

Ob rvations in other tissue

We have obt ined simil r conclusions for the localization of nion-sensitive ATPas in v rious other tissues The b ence of a plasma membrane located anion-sensitive ATPase in rainbow trout gill (9) appears to exclude a rol of the enzyme in cellular anion transport in th gill The results obtained f r the brush border membrane fraction and other sub-cellular fractions of rabbit kidney (19) seem to argue against the postu-lated role of the enzyme in th bicarbonat reabsorption of the kidney proximal tubule (20 21) The enzyme activity from at pancreas whi h was found to be primarily located in a mitochondri l (15 min 15 000 x g) frac-tion f this tissue (10 22) initially appe red to be insensitive towards oligomycin in gr ement with the findings of Simon et l (23) This in-sensitivity could however be attributed to the high (phospho)lipase activity in the pancreas since removal of Ca^2 by EDTA during fractiona-tion leads t complete inhibition of the enzyme

Finally the presence of the enzyme in the r bbit erythrocyt membrane has been confirmed (10 24) However it characteristics differ from those of th enzyme activity in all oth r tissues tudied Calcium stimu lates the erythrocyte acti ity wheres EGTA ruthenium ed and chlor promazine are inhibitory Hence we conclud that th activity is part of the Ca-Mg-ATP se system of the erythrocyt membrane

Concluding remarks

We are f ed to conclud from our experiments that rabbit gastri mucosa rainbow trout gill r bbit kidney and rat pancreas contain only a mitochondri l anion- ensitive ATP but not a plasma membrane located activity Th nion-sensitive ATPas activity p sent in the rabbit rythrocyte memb ane i pa t f th Ca-Mg a tivated ATP sy tem Thes findings appear t rul out function f the anion- ensitive ATPase in the transport of anions acros the pl sma memb ane in th ti sues

Another candidat f r a l in g tric ecr tion (Na-K)ATPas p- pears t be only indirectly involved (25 26) The i now ome evidence that an ouabain-insen itive potas ium-stimul ted ATPase tivity reported t be located in th gastri muco al pl sma membrane (11 27) may be involved in acid secr tion (28) Thi tivity i diff rent from the (Na-K)-ATPas as hown by phosphorylation experiment with ATP (29) Mi ro omal plasma membrane vesi les containing this enzyme activity hav been puri fied (30) and have been hown t exchange H^+ i ns against K^+-ions at the expense f ATP (28 31 33; ee lso paper by Ray and T gue Lewin t l Saccomani t al Mich langeli and Proverbi and Scholes in this volume) Furthe tudies will have to prove that thi potas ium-stimulated ATPas activity can indeed play the suggested rol in ga tri ecr tion and if so how it ope ates in the pari t l all

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Some New Aspects of Anion Pumping by the Turtle Bladder

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ABSTRACT In short-circuited turtle bladders bathed on both surfaces by identical HCO_3-rich choline Ringer media with Cl the serosal addition of SITS (10^{-4}M) caused a nullification in the PD and I_{sc} but a finite reabsorption of Cl remained In the absence of exogenous Cl, the same addition of SITS caused a reversal in the PD and I_{sc} which can be attributed to an active electrically-conductive secretion of HCO_3 This secretion stimulated by theophylline does not necessarily occur co-temporally with the known reabsorption of HCO_3 by this epithelium.

It has been established that the urinary bladder of the fresh water turtle (Pseudemys scripta) dilutes and acidifies the luminal fluid by means of three discrete pump mechanisms for the reabsorption of Na Cl and HCO_3 (1 7) Recent data to be shown here indicate the presence of yet another pump mechanism presumably for the secretion of HCO_3 This secretion has been found in short-circuited turtle bladders bathed on both surfaces by identical HCO_3-rich choline media (in the presence and in the absence of exogenous Cl) after exposure of the serosal surface of these bladders to the disulfonic stilbenes (SITS and DIDS) In the absence of exogenous Cl such data constitute strong evidence for the electrogenicity of this HCO_3 secretion. Some of the experimental details and physiological implications of the findings are discussed below

Methods used described in detail previously (3) entail the simultaneous mounting of two sections of a single bladder in a double-

barreled Rehm-Ussing chamber The hemi-bladders were interposed between identical Na-free (choline) Ringer solutions with or without Cl or HCO_3 (as specified below) and then short-circuited Ouabain (10^{-4} M) was added to the serosal fluid and amiloride (10^{-5}M) to the mucosal fluid The transepithelial I_{sc} open-circuit potential (PD) and resistance were then monitored before and after the serosal addition of SITS

Table 1 SITS-induced changes in anion transport-related parameters of bladders in (Cl + HCO_3)-containing Ringer

Period	PD mV	I_{sc} (ua/1 5 cm^2)	I_{ms}^{Cl}
	Group I (n=8) I_{ms}^{Cl} decreased		
Control	50 1 ± 6 4	39 3 ± 8 8	31 5 ± 8 5
After SITS	0 2 ± 1 2	0 4 ± 1 1	17 0 ± 3 8
	Group II (n=8) I_{ms}^{Cl} unchanged		
Control	31 8 ± 6 2	28 2 ± 7 5	12 9 ± 2 6
After SITS	0 1 ± 0 5	0 0 ± 0 5	11 1 ± 1 9

Means of the individual percentage changes were statistically significant for all parameters of both groups with the exception of I_{ms}^{Cl} in group II

Table 1 shows that after the addition of SITS to the serosal fluid of bladders bathed on both surfaces by identical (Cl + HCO_3)-containing choline Ringer media the transepithelial PD and I_{sc} were nullified while the forward (or m-to-s) flux of chloride (I_{ms}^{Cl}) and the calculated net chloride reabsorption (not shown) remained finite Nevertheless I_{ms}^{Cl} was significantly decreased in some bladders (SITS-sensitive chloride

flux group) but not in others (SITS-insensitive chloride flux group) The effect of SITS on the backflux (or s-to-m) flux of chloride (I_{sm}^{Cl}) was no different than that expected from aging of the in-vitro bladders (e g the mean values of I_{sm}^{Cl} were 4 5 ± 0 9 ua before SITS and 6 7 ± 1 1 ua after SITS in 29 other half-bladders)

The finite net reabsorption of chloride ion in the electrically-silent SITS-treated bladders requires the concomitant transepithelial flow of another ion. At present the active secretion of HCO_3 appears to be the most probable identification of the process that balances this residual net reabsorption of Cl after SITS This claim is based in part on previously reported data of Leslie et al (8) and Oliver et al (9) and in part on our recent data which are as follows

(i) SITS produced no significant changes in the m-to-s or s-to-m flux of $^{35}SO_4$ in 9 experiments (ii) Neither the control levels of PD and I_{sc} nor the nullification of these parameters after SITS was altered in 3 experiments in which the bladders were bathed by (Cl + HCO_3)-containing choline Ringer solutions devoid of exogenous K Mg and phosphate on the serosal surface and devoid of all of these and Ca on the mucosal surface Essentially the same findings before and after SITS were found in 2 experiments in which Tris or K was substituted for the choline in both bathing fluids

Thus by elimination the active secretion of HCO_3 appears to be the process that balances the active reabsorption of Cl to produce the electrically-silent state of the SITS-treated bladders under these bathing conditions

If this HCO_3 secretion were part of a non-conductive (electroneutral) mechanism that mediates a one-for-one exchange of HCO_3 secretion for Cl

reabsorption then the magnitude of the I_{sc} could not exceed that of the net HCO_3 reabsorption (or proton secretion) in bladders bathed by Cl-free, HCO_3-rich choline media and the serosal addition of SITS should be followed by the reduction of this I_{sc} to or near zero levels. If on the other hand this HCO_3 secretion were an electrically-conductive flow driven by an independent electrogenic pump the I_{sc} would be equal to the algebraic sum of the net reabsorption and net secretion of HCO_3. In this case the addition of SITS might well be followed by a reversal in the orientation of this I_{sc}.

Table 2. Mean values of PD and I_{sc} before and after SITS-treatment of bladders in Cl-free HCO_3-containing choline Ringer media (n=7)

Period	PD mV	I_{sc} ua/1.5 cm^2
Control	26.6 ± 6.7	14.6 ± 4.3
After SITS	-7.3 ± 6.3	-3.5 ± 0.4

Minus sign denotes that the mucosa is electronegative to the serosa. Changes shown are statistically significant (P <0.001)

Table 2 shows that the serosal addition of SITS was followed by a sustained reversal in the orientation of the PD and I_{sc} in 7 ouabain-treated bladders each of which was bathed on both surfaces by an identical Cl-free HCO_3-rich choline Ringer solution. Before SITS the PD and I_{sc} were in the orientation (m positive s) consistent with active HCO_3 reabsorption (or H secretion). One hour after the addition of SITS the PD and I_{sc} reached zero then reversed in orientation so that the mucosal fluid became electronegative to the serosal fluid and remained so for the next 2 to 3 hours.

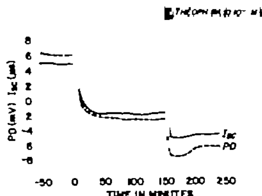


Figure 1 PD and I_{sc} versus time before and after the serosal addition of SITS and then theophylline to a bladder bathed in Cl-free HCO_3-rich media. Sign conventions are those defined under Table 2

Figure 1 shows the effect in a selected experiment of this group Also shown is the fact that the addition of theophylline (10^{-2}M) to both bathing fluids was followed by a doubling in the magnitude of the reversed PD and I_{sc} in this and in 4 other similarly treated bladders

This sustained asymmetrical electrical state (m negative s) constitutes sufficient evidence for the active and electrically-conductive translocation of some ion most probably for the secretion of HCO_3 (in view of the data on ion substitution described above) It should be noted that no matter what its ion composition a finite short-circuiting current cannot be identified solely as an electroneutral process (e g ion exchange or ion-pair translocation)

Figure 2 is an electrical circuit model the behavior of which simulates that of open- and short-circuited bladders in Na-rich and Na-free media. Like the net transepithelial fluxes of Na Cl and HCO_3 in the bladder the 'trans-network' flux of ion-selective currents in the model depends upon the orientation as well as the magnitude and selectivity of the driving forces (E terms) and upon the magnitude and selectivity of the conductive elements (g terms) in its apical and basal-lateral networks (denoted A[and BLM)

reabsorption then the magnitude of the I_{sc} could not exceed that of the net HCO_3 reabsorption (or proton secretion) in bladders bathed by Cl-free, HCO_3-rich choline media and the serosal addition of SITS should be followed by the reduction of this I_{sc} to or near zero levels If on the other hand this HCO_3 secretion were an electrically-conductive flow driven by an independent electrogenic pump the I_{sc} would be equal to the algebraic sum of the net reabsorption and net secretion of HCO_3 In this case the addition of SITS might well be followed by a reversal in the orientation of this I_{sc}

Table 2 Mean values of PD and I_{sc} before and after SITS-treatment of bladders in Cl-free HCO_3-containing choline Ringer media (n=7)

Period	PD mV	I_{sc} ua/1 5 cm^2
Control	26 6 ± 6 7	14 6 ± 4 3
After SITS	-7 3 ± 6 3	-3 5 ± 0 4

Minus sign denotes that the mucosa is electronegative to the serosa Changes shown are statistically significant (P <0 001)

Table 2 shows that the serosal addition of SITS was followed by a sustained reversal in the orientation of the PD and I_{sc} in 7 ouabain-treated bladders each of which was bathed on both surfaces by an identical Cl-free HCO_3-rich choline Ringer solution Before SITS the PD and I_{sc} were in the orientation (m positive s) consistent with active HCO_3 reabsorption (or H secretion) One hour after the addition of SITS the PD and I_{sc} reached zero then reversed in orientation so that the mucosal fluid became electronegative to the serosal fluid and remained so for the next 2 to 3 hours.

counted for and represented in physical terms by the operations of the electrical model in Figure 2

When the bladder is treated with ouabain or amiloride or bathed by Na-free media containing Cl and HCO_3 the Na parameters become vanishingly low (i e $g_{Na,a} = g_{Na,b} = 0$ $I_{Na} = 0$) m becomes electropositive to s and the I_{sc} can be formally represented by the equation

$$I_{sc} = G_t\ PD_{ms} \qquad 1)$$

(by definition) where G_t is the transepithelial conductance and PD_{ms} the open-circuit potential It can then be shown that PD_{ms} under these conditions is equal to the sum of the products of partial ion conductances ($\bar{g}$ terms) and electromotive forces (E terms) in the apical membrane or

$$I_{sc} = (G_t)\ (\bar{g}_1E_1 - \bar{g}_2E_2 + \bar{g}_3\ E_3) \qquad 2)$$

where the subscripts 1 2 and 3 denote the electrical force terms for HCO_3 reabsorption HCO_3 secretion and Cl reabsorption respectively From Kirchoff s law it follows that

$$I_{sc} = I_1 - I_2 + I_3 \qquad 3)$$

Before SITS the magnitude of $I_1 + I_3$ must exceed that of I_2 as is shown by the observed orientation of I_{sc} and PD_{ms} After SITS under these conditions the PD and I_{sc} vanish but the net reabsorption of Cl remains finite and balanced by an apparent net secretion of HCO_3 $(I_2 - I_1)$ This is because the observed I_{sc} and PD vanish after SITS which means that the algebraic sum of all driving forces and ion-selective currents must also be equal to zero and consequently

$$\bar{g}_3E_3 = \bar{g}_2E_2 - \bar{g}_1E_1 \qquad 4)$$

and

$$I_3 = I_2 - I_1 \qquad 5)$$

When all exogenous Cl is removed the bladder [illegible] ed on both surfaces by identical HCO_3-rich [illegible] de [illegible]



are vanishingly low (i e $\bar{g}_3E_3 = 0$ $I_3 = 0$) and the mucosa is electropositive to the serosa ($PD_{ms} > 0$) which leads to the following I_{sc} functions

$$I_{sc} = (G_t)\ (\bar{g}_1E_1 - \bar{g}_2\ E_2) \qquad 6)$$

and

$$I_{sc} = I_1 - I_2 \qquad 7)$$

Before SITS $PD_{ms} > 0$ and hence the magnitude of $\bar{g}_1E_1$ must exceed that of $\bar{g}_2E_2$ and hence the magnitude of I_1 exceeds that of I_2 After SITS under these Cl-free conditions the orientation of the PD and I_{sc} reverses so that m becomes and remains electronegative to s i e $PD_{ms} < 0$ in the SITS-treated state of these bladders Since this reversed PD is finite the algebraic sum of the driving forces must be finite and the sum of the ion currents must also be finite However in contrast to the control state the magnitude of $\bar{g}_2E_2$ must exceed that of $\bar{g}_1E_1$ and the magnitude of I_2 exceeds that of I_1 In other words the net secretion of HCO_3 must exceed the net reabsorption in order to be physically consistent with the observed orientation of PD and I_{sc} in SITS-treated bladders under these Cl-free conditions Neither this nor any other sustained and finite level of PD or I_{sc} can be simulated by the sole action of an electroneutral mechanism that mediates a one-for-one exchange of HCO_3 for Cl reabsorption (8 9 18)

The assumption that the single primary action of SITS and DIDS is to decrease the anion-selective conductance ($g_{anion\ b}$) of the basal-lateral membrane (15 16) is consistent with the reduction in the PD and I_{sc} to near-zero levels in bladders bathed by Cl rich HCO_3-rich choline media But this alone cannot account for the reversal in orientation of the PD and I_{sc} in bladders bathed by Cl free HCO_3-rich choline media and consequently one is forced to invoke secondary changes in the electrical

parameters of the apical membrane

For example the concentration of cellular HCO_3 would be expected to increase subsequent to the SITS-induced lowering of $g_{anion\ b}$ in a bladder that had been translocating HCO_3 from m to s prior to the serosal addition of SITS This increased HCO_3 concentration in the fluid bathing the inner surface of the apical membrane would increase the magnitude of the inherent EMF (E_2) and internal conductance (g_2) of the putatively electrogenic pump for HCO_3 secretion and decrease that of the inherent EMF (E_1) of the pump for HCO_3 reabsorption The observed SITS-induced reversal of PD and I_{sc} requires that these apical membrane changes continue until the magnitude of $\bar{g}_2E_2$ exceeds that of $\bar{g}_1E_1$ At this point the net direction of the HCO_3 pumping across the apical membrane is from cell to mucosal fluid which requires that the net transepithelial flow be directed from the serosal to the mucosal fluid Thus the model can simulate the electrically-silent state ($PD_{ms} = 0$) as well as the electrically-asymmetrical state ($PD_{ms} < 0$) of SITS-treated bladders under each of the Na-free bathing conditions imposed.

The simultaneous presence of acidification and alkalinization mechanisms in the bladder does not necessarily mean the simultaneous operation of these mechanisms this would be inefficient with respect to maintaining the acid-base balance of the body fluids On the other hand the mucosal acidification mechanism could be activated under one set of conditions (e g acid-ash diet) and the mucosal alkalinization mechanism activated under another set of conditions (e g alkaline-ash diet) In this connection McKinney and Burg have recently reported the activation of a HCO_3 secretion into the single renal collecting tubes of rabbits that had been ingesting alkaline-ash diets (19)

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Factors Affecting Pancreatic Anion Secretion

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ABSTRACT The ff cts of several manoeuvres which affect pancreatic fluid and lectrolyte secretion have been tudied in the isolated perfused cat pancreas There was found to be no unique requirement for perfusate bicarbonate; several weak organi acid anions could substitute successfully but none were as effective as bicarbonate in promoting fluid secretion Fluid secretion depended on th concentration of buffer anion in perfusate in th case of cetate and also on th pH of perfusion fluid. Distribution of metabolically produced CO_2 between perfusate and pancreatic juice was found to be dependent on the pH of the perfusion fluid. Certain osmotically active substances when added to th perfusion fluid affected both fluid secretion and cation and anion concentrations in th juice Two groups of substance are described with differential effects on anion concentr tion of th juice

Introduction

Th bulk of bicarbonate which appears in pancreatic juice is derived from blood only a small amount (6%) arising from th pancre tic cells (1 2 3 4) However although the pancre ti secretory te is linearly related to xtracell lar fluid bicarbonate concentration (1) it has been shown that in th isolated cat pancreas (2 4) and in the in itro rabbit pancreas (3) th re is o unique requirement for bicarbonate. Certain weak organi acids (2) and weak organic acid anion (5 6 4) can substitute fo bicarbonate and promote lectrolyte and fluid secretion to varying degrees though none are as ff ctive as bicarbon te.

Th mechanism of bicarbon te secretion is till not clear though several models h ve been proposed (1 2 6) The fact that extracellular fluid bicarbonate can be ubstituted by range of buff anions

supports the concept that H^+ or OH is transported primarily (7) though the site of this process is still unknown

The purpose of this study was to confirm and extend the substitution experiments of Swanson and Solomon (6) using an isolated saline perfused preparation of the cat pancreas (8) and also to investigate the effects of varying the pH of the perfusion fluid on fluid and electrolyte secretion In addition the effects of adding several inert but osmotically active substances to th perfusion fluid on pancreatic secretion rate and electrolyte content of pancreatic juice are reported

Methods

The isolated pe fused pancreas of the cat previously described in detail (8) was used in all experiments The prepar tion was modified lightly in that after removal from the animal the pancreas was maintained in a specially-constructed thermostatically controlled plastic box The gland was perfused with Krebs bicarbonate saline (iso-osmolal with cat plasma) except where indic ted in the text and timulated to secrete maximally by infusion of secretin Pancreatic juice was collected at ten minute intervals in tared plastic tubes and its mas determined gravimetri ally Samples of juice were analysed fo Na^+ K^+ Cl and HCO_3^- conce tr tion and osmolality where appropriate Determinations of organic anions in the juice was by diff rence from a knowledge of th above In the pH experiments the pH of the inflow and outflow pe fusion fluids was continously monitored.

Results

Eff ct of substituting perfusion fluid bicarbonate with cetate

Th effects of increasing perfus te acetate concentration ove a range from 0 to 40 mM are shown in Figure 1. In all cases iso-osmolality was maintained by varying pe fusate NaCl concentration and pH was kept constant at 7 4 As can be seen th secretory rate increased lmost line rly up to a perfusate acetate concentration of 25 mM but t concentrations above 25 mM secretory r te began to pl te At equimolar concentrations of 25 mM acetate was f und to be only about 45% as effective as bicarbonate in promoting fluid secretion Maximum fluid secretion was obtain d t an acetate concent tion of about 50-60 mM but even then only re ched about 60% of that obtained with 25 mM bicarbonate

The ffects of 25 mM acetate on anion composition of the juice are shown in Figure 2 Th time course of fluid s cretion was found to be

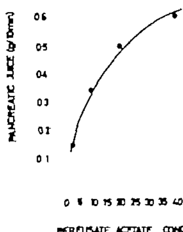


Fig 1. The results of single experiment to illustr te the effects of varying perfusate acetate concentration on pancreatic cretory rate I ll cases iso-osmolality was maintained by varying NaCl concentr tion

imilar to that when bicarbonate was th buffer taking about 40 minutes to reach maximal secretory rate after initi tion of a cretin infusion Again th maximum secretory rate obtained of 0 5 g 10 min [1] was about 50% of that obtained with 25 mM bicarbonate in th perfusion fluid. The most striking feature of th anion composition of the juice was th small amount of bicarbonate appearing in the juice (5-10 mM) even though no bicarbonate was present in th perfusion fluid Chloride ion appeared in th juice t concentration of about 60 mM which is similar to that found at equivalent secretory r te with bicarbonate as th buff r Th juice acetate concentration was also equivalent to the expected bicarbonate concent tion of comparable secretory ate

Effects f other weak organic acid anions

A comparison of the ffect of perfusate bicarbonate replacement with

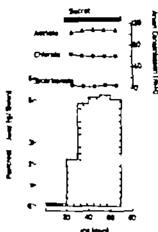


Fig 2 Effects of substitution of perfusate bicarbonate with 25 mM cetate on pancreati juice output and anion concentr tion of th juice when timulated maximally by ecretin for the duration of th black ba

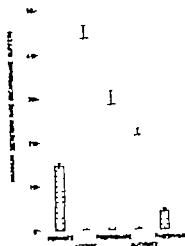


Fig 3 A comparison of the effectiveness of substituting perfus te bicarbonate with 25 mM concentrations of various weak organic acid anions in promoting pancreatic fluid secretion Fluid secretion is expressed as a % of the maximum secretory rate obtained with 25 mM bicarbon te in the perfusate

25 mM concentrations of acetate formate propionate and butyrate at pH 7 4 is shown in Figure 3 Of those tested cetate was the most ffective in supporting pancreatic fluid secretion being about 44% as ffective as an equimolar concentration of bicarbonate Propionate (29% as effective) butyrate (21%) and formate (15%) were also found to support fluid secretion but to a lesser degree By comparison phosphate buffer supported secretion to only a small extent *less than 5% of the maximum* cretory rate obtained with bicarbonate buffe

Eff cts of changing perfusion fluid pH

A number of experiments were also performed with perfusion fluid contai ing 25 mM acetate (replacing bicarbonate) in which the pH of the pe fusion fluid entering the gland was varied over range 6 0 to 8.0 by

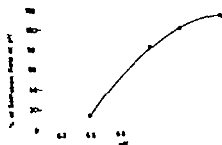


Fig 4 The results of ingle experiment to illustr te the eff ct of changing perfus te pH on pancreatic secretion r te expressed as percentage f the secretion rat observed t pH 7 4

addition of small amounts of HCl or NaOH The results are shown in Figure 4 where the secretion rate is xpress d as a percentage of that obtained at pH 7 4 As the pH of the perfusion fluid was decreased below 7 4 th secretion rate fell almost linearly and secretion ceased t perfusate pH of about 6 0. At perfusate pH above 7 4 there was little increase in secretion rate

A number of experiments were also performed in which the pH of th inflow perfusion fluid (25 mM acetate replacing HCO_3^-) was decreased in tep-wise fashion from 7 4 to 6 4 and th pH of th ffluent perfusion fluid was monitored Pancre ti secretory rate and bicarbonate ion concentration of th juice were also measured. Thes results are presented in Figure 5 and show cl arly that t an inflow pH of 7 4 th outflow pH was always les (approximately 6 9) As th inflow pH was decreased further so th outflow pH als fell but to a lesser degree such that th difference between the two became less At an inflow pH of 6 6 this ituation was reversed and although the outflow pH also fell it became slightly greater than the inflow At an inflow pH f 6 4 the diff rence was more marked and th outflow pH was 0.2 units higher than the inflow

As observed previously small amount of bicarbonate also appeared in pancre ti juice (< 10 mM) and it was noticeabl that as th pH of th inflow was decreased, there was a concomitant fall in HCO_3^- concentration of th juice so that t an inflow pH of 6 4 juice HCO_3^- concentration was almost zero

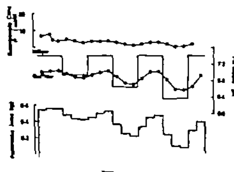


Fig 5 The ffect of tep-wise reduction of perfus te pH on pancreatic juice out put (shaded columns) the pH of th effluent perfus te (outflow) and juice bicarbonate concentration

Effect of Hyperosmolality

Any theory of electrolyte secretion should be able to account for the effect on secretion of subjecting th gland to an osmotic load. A large number of apparently inert substance was investigated and these produced two groups of compounds with marked differential effects on anion conce tration of the pancreatic juice In all experiments the substance under study was added to normal perfusion fluid in a concentration of 100 mM. In ch instance the effects on secretory rat and electrolyte content of the juice were determined by comparing the control period immediately before exposure of the gland to the osmotic load with that of th second collection period during which the gland was perfused with hyperosmolal solution Of the substances tested four sucrose arabinose xylose and sorbitol (Table 1) inhibited secretion by about 70% These substances also increased the anion and cation concentration of the juice so th t equiosmolality between perfusate and juice was maintained within experimental limits Of the cations K^+ concentration changed very little (about 1 mM) while Na^+ concentration was increased markedly (about 50 mM) With the anion both Cl and HCO_3 concentrations were significantly increased but in all experiments the change in Cl^- concentration was greate than that in HCO_3^- concentration such that th r tio $\Delta Cl / \Delta HCO_3$ was always greater than unity The s cond group of substances urea

Table 1 Eff cts of group I and group II substance on pancre tic s cretion r te and changes in el ctrolyte composition of the juice Compounds were added to perfusion fluid at concentr tion of 100 mM

Group I compounds						
Compound	Reduction Sec. Rate	ΔNa	ΔK	ΔCl	ΔHCO_3	$\Delta Cl/\Delta HCO_3$
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Group II compounds						
Compound	Reduction Sec. Rate	ΔNa	ΔK	ΔCl	ΔHCO_3	$\Delta Cl/\Delta HCO_3$
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

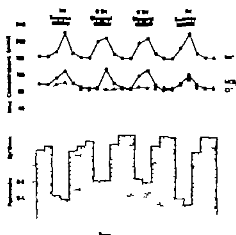


Fig 6 The results of a ingle experiment to compare th ffect of addition of 100 mM concentration of glycerol and ucrose to perfusion fluid on pancreatic secretion rate (tippled columns) and electrolyte composition f the juice

thiourea and glycerol which were more permeable inhibited fluid secretion by pproximately 12% 36% and 40% respectively (Tabl 1) Again both anion and cation concentrations of the juice were increased and again th cation change was chiefly N^+ However with the anions the greatest change was seen in th HCO_3 concentration such that for these compounds th $\Delta Cl/\Delta HCO_3$ ratio was lways less than unity Of these substances glycerol had the most marked ffect in changing HCO_3^- concentration of th juice Th results of single experiment in which the effects of equal hyperosmolal solutions of sucrose and glycerol were compared in the ame animal are shown in Figure 6 Both sucrose and glycerol inhibited fluid secretion ucrose being more ffective than glycerol (70% inhibition compared to 50%) and both substances increased anion and cation concentrations of th juice Neither substance had an ffect on K^+ concentration but both ignificantly increased Na^+ concentration In contrast with th anions sucrose increased both Cl and HCO_3 concentrations while glycerol increased only th HCO_3 concentration of th juice

Discussion

The results confirm that in the isolated perfused cat pancreas there is n unique requirement for bicarbonate as th buffer in extracellular fluid. Several weak organi acid anions can substitute successfully f r bicarbonate though none are as eff ctive in promoting fluid secretion Of those tested acetate was th most ffective though even at perfusate concentrations of 50-60 mM, cetat produced only about 60% of th maximal secretory rate observed with 25 mM bicarbonate Th other anions

propionate formate and butyrate were less effective

These results are similar to those of Swanson and Solomon (6) though we have not been able to confirm that acetate substitution is as eff ctive as bicarbonate in promoting electrolyte and fluid secretion

It was also found that a small amount of bicarbonate was secreted in the juice even when bicarbonate was omitted from the perfusion fluid This can only have come from the pancre tic cell and provides additional evidence to the view that the bulk of pancre tic juice bicarbonate is of extracellular origin Th bicarbonate concentration of the juice was found to be in th order of 8-10 mM indicating that only about 6% of normal juice bicarbonate arises from cellular metaboli m.

Th relationship of secretory rate with acetate concentration of the perfus te on the one hand and perfusion fluid pH on th other also confirms the observations of Swanson and Solomon (6) in th in vitro rabbit pancreas During the experiments in which the pH of the perfusate was varied it was observed that the normal relationship in which the pH of the effluent perfusion fluid was lower than that of the inflow was reversed when th inflow pH was about 6 6 or lower The concentration of metabolic CO_2 appearing in the juice was determined by the pH of the inflow perfusate The reversal effect on pH noted above could probably be due to diversion of the CO_2 from juice to perfusate so increasing the pH of the latter

Osmotic loads when added to the perfusate caused a reduction in secretory rate in proportion to the degree of permeability of th test substance The osmolality of the secretion and perfusate unde all conditions was equal within experimental error Group 1 substances sucrose arabinose xylose and sorbitol (Table 1) were found to be relatively impermeable reducing the secretion rate by about 70% and increasing th concentration of all the ionic components of the pancreatic juice The cation change was almost entirely due to an increase in concentration of sodium whereas in the case of th anions chloride concentration was increased pref rentially to that of bicarbonate so that $\Delta Cl/\Delta HCO_3$ was always greater than unity In the case of th Group II (Table 1) substances ure thiourea and glycerol these compounds inhibited secretion by about 12% 36% and 48% respe tively The ionic concentrations also increased but did not account for th measured osmolality of the juice consequently one of the test

substances must have entered the juice to an extent determined by their permeability The anions however exhibited a different behaviour to that de cribed in response to osmotic loads of group I substances In the case of group II ubstances they caused preferential increas in bicarbonate so that $\Delta Cl^-/\Delta HCO_3^-$ was lways less than unity

Th ite at which these anion changes are brought about is uncertain but could be t the locus of th primary secretion by ff cts on H^+ ion secretion or it could be lower in th duct system where Cl^-/HCO_3^- exchange i known to occur

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   J Physiol 196 133-149

The experimental determination of either value is problematic The magnitude of t membrane potential of Ehrlich cells as determined by different methods varies re- rr kably Micropuncture leads to 25 mV inside negative (2), possibly an underesti or due to damage of the cell membrane during the impalement of the electrode lations from the distribution of lipid soluble cations (3) or from the fluorescence ng of cyanine dyes (4 5) give values of 40 60 mV inside negative possibly estimation due to accumulation of these substances in mitochondria

he H^+ ions distributed according to the membrane potential the acidity of the sm would exceed that of the extracellular fluid by at least o 4 pH units assu- membrane potential of at least 25 mV, inside negative

ce the direct determination by micropuncture has not been employed success lly with Eh lch cells so far the pH difference between cell and medium is usually calculated from the distribution ratio of certain weak electrolytes which penetrate the cell membrane exclusively in the uncharged form

If for instance the weak acid AH is uncharged and permeates whereas the conjugated base A^- does not the intracellular pH is

$$pH_i = pH_e + \log(A_i^- / A_e^-)$$

If on the other hand the weak base B is uncharged and permeates whereas the conjugated acid BH^+ does not the intracellular pH is

$$pH_i = pH_e + \log(BH_e^+ / BH_i^+)$$

It should be pointed out however that the determination with an acid AH and a base B gives the same value for the intracellular pH only if there are no compartments of different pH values inside the cell (6) Otherwise the pH determined with B must always be more acid than that determined with AH (In the appendix the mathematical derivation is presented)

Fig 1 shows schematically a cell which contains two compartments of equal size one with pH 8 0 and the other with pH 6 0 whereas the extracellular pH is 7 0 Relative to the whole space there is a fivefold accumulation of the base B as well as

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   J Physiol 196 133-149

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Is There an Active Proton Pump in Ehrlich Cells?

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ABSTRACT: Investigations with DMO nd benzylamine as pH indicators show that there are compartments with different pH inside the Ehrlich cell In respi ing cells compartments exist slightly lkaline in respect to the medium while others are by about 1 pH unit more acid The influence of different inhibitors on the intracellular pH distribution leads to the assumption that the more alkaline compartment represents the cytoplasm One has to assume that there exists a mechanism pumping protons out of the cell since otherwise a membrane potential inside negative would lead to an cidic cytoplasm

The metabolic production of acid of animal cells would lead to progressiv acidification of the cell interior if the egress of the H^+ ions through the cell membrane were not possible Accordingly a continuous delivery of acid from Ehrlich cells can be observed by titration at pH stat condition (1) This process is not influenced by ouabain but totally suppressed by anoxi From this O_2-dependence however it does not follow that the transport mechanism for H^+ ions is energy-dependent since the rate of titration might be limited by the metabolic acid production in the cell and not by the capacity of the proton transport system if it exceeds that of the acid production Hence it is not clear whether the H^+ ions leave the cell passively down their electrochemical potenti l gradie t or whether they are extruded by primary or secondary active transport

In the case of active transport the proton concentration in the cytoplasm should be lower than calculated from the Nernst distribution To test thi one must know both the lectrical pote ti l difference ($\Delta\Psi$) and the pH difference (ΔpH) betwee cytoplasm and extracellular fluid

The experimental determination of either value is problematic The magnitude of the membrane potential of Ehrlich cells as determined by different methods varies remarkably Micropuncture leads to 25 mV inside negative (2) possibly an underestimation due to damage of the cell membrane during the impalement of the electrode Calculations from the distribution of lipid soluble cations (3) or from the fluorescence quenching of cyanine dyes (4 5) give values of 40 – 60 mV inside negative possibly an overestimation due to accumulation of these substances in mitochondria

If the H^+ ions distributed according to the membrane potential the acidity of the cytoplasm would exceed that of the extracellular fluid by at least o 4 pH units assuming a membrane potential of at least 25 mV inside negative

Since the direct determination by micropuncture has not been employed successfully with Ehrlich cells so far the pH difference between cell and medium is usually calculated from the distribution ratio of certain weak electrolytes which penetrate the cell membrane exclusively in the uncharged form

If for instance the weak acid AH is uncharged and permeates whereas the conjugated base A^- does not the intracellular pH is

$$pH_i = pH_e + \log(A_i^- / A_e^-)$$

If on the other hand the weak base B is uncharged and permeates whereas the conjugated acid BH^+ does not the intracellular pH is

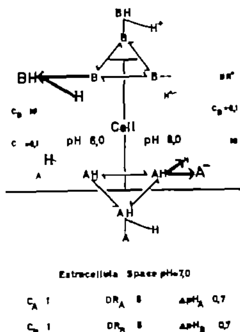
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It should be pointed out however that the determination with an acid AH and a base B gives the same value for the intracellular pH only if there are no compartments of different pH values inside the cell (6) Otherwise the pH determined with B must always be more acid than that determined with AH (In the appendix the mathematical derivation is presented)

Fig 1 shows schematically a cell which contains two compartments of equal size one with pH 8 0 and the other with pH 6 0 whereas the extracellular pH is 7 0 Relative to the whole space there is a fivefold accumulation of the base B as well as

Fig 1: Heterogeneity of intracellular pH

Schematic representation of the distribution of a weak acid AH and weak base B as used for determination of intracellular pH



for the acid AH due to a tenfold accumul tion of the base in the acid compartment and a tenfold accumulation of the acid in the alkaline compartment The intracellular pH calculated from these two distribution ratios is 6 3 for the base and 7 7 for the acid respectively Thus the value determined with the base is approximately that of the cid compartment while the acid monitors rather the alkaline compartment

This fact that the determination of the intracellular pH by an cid or by a base yields different results is presented i nother way on Fig 2 The pH difference ΔpH_a determined by the distribution of the acid is plotted versus ΔpH_b that determined by the distribution of a base Thus each poi t in this plot represents two pH measurements: the ordinate giving the pH as determined by the acid (ΔpH_a) and the abscissa the pH as determined by the base (ΔpH_b) Applied to our above model system (Fig 1) this plot yields a point whose location clearly indicates that one of the two compartments is more acid than the medium corresponding to a negative ΔpH_b whereas the othe is more alkaline than the medium corresponding to a positive ΔpH_a In thi plot any such system will be represented by points exclusively located abov or on the line $\Delta pH_a = \Delta pH_b$ Poi ts on the line represe t homogeneous systems Points above this line represent inhomogeneous systems The distance of the point from this line is a

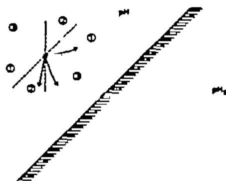


Fig 2: Heterogeneity of intracellular pH

Schematic representation of intracellular pH changes as measured by a weak acid and a weak base

measure of the heterogeneity of the system Points below this line are forbidden since the pH determined with the base can only be smaller or equal to the pH determined with the acid The neighborhood of such a point can be di ided into three regions Variation of the pH in the acidic compartment affects mainly the distribution of the base and should therefore shift the point into region 1 Variations of the pH in the alkaline compartment affects mainly the distribution of the acid and should shift the point into region 2 Variations of the pH in both intracellular compartments in such a way that the intracellular pH differences remain uninfluenced results in a shift along the dotted line parallel to the line $\Delta pH_a = \Delta pH_b$ representing homogeneous systems If the shift is into region 3 the pH values of the two compartments are varied in opposite direction

Fig 3 shows an experimental example for the variation of the pH in the acid compartment of Ehrlich cells The cells were incubated with various benzylamine concentrations up to 10 mM A control without addition of unlabelled benzylamine shows that there are remarkable pH differences inside the cell as the point lies far remote

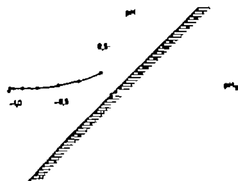
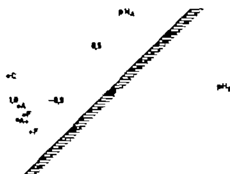


Fig 3: Influence of increasing benzylamine concentrations on the intracellular pH

Transcellular pH difference calculated from the DMO distribution (ΔpH) is plotted versus that calculated from the benzylamine distribution (ΔpH_b) Benzylamine varied from 0 to 10 mM

Fig 4: The effect of various inhibitors on the intracellular pH

Plot as in Fig 3
C: control
A: 6 μM antimycin A
F: 12 μM FCCP
V: 31 μM valinomycin



from the line representing homogeneous systems With rising benzylamine concentration one sees that the distribution ratio of the acid ^{14}C 5 5 -dimethyloxazolidin-2 4-dione (DMO) is barely affected while the distribution ratio of the base ^{14}C-benzylamine is diminished This result is to be expected since the H^+ ions in the acidic compartments are gradually neutralized by benzylamine

In the present context the question is what is the acidity of the cytoplasm of the Ehrlich cells under aerobic conditions As has been shown before acidic and basic probes give quite different pH values with these cells thus indicating two major compartments with distinctly different pH (1) It is likely that the cytoplasm owing to its relative size refers at least to a major part to one of these compartments but there is no direct indication whether to the more acid or to the more basic one Since the mitochondria owing to thei proton pump tend towards lkalinity aerobically they are likely to form part of the more basic compartment I our determination If the cytoplasm would be one pH unit more acid than the medium according to the benzylamine distribution it is not necessary to assume any other mechanism for the extrusion of H^+ ions from the cell than leak

The addition of some inhibitors of mitochondri l metabolism such as antimyci A valinomyci and carbonyl cyanide p-trifluoromethoxy-phenylhydrazon (FCCP) causes indeed acidification of the more basi compartment as can be concluded from the shift of the plotted point almost parall l to the ΔpH_a-axis (ordinate) into the acid region (Fig 4) The slight deviation to the right could the indicate a reduced proton gradient between cytoplasm and medium the result of the depolarisation of the plasma membrane owing to the depletion of ATP neccessary for the operation of the lectroge ic Na K-pump (1)

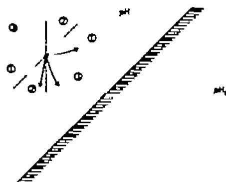


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Schematic representation of intra cellular pH changes as measured by a weak acid and a weak base

measure of the heterogeneity of the system Points below this line are forbidden since the pH determined with the base can only be smaller or equal to the pH determined with the acid The neighborhood of such a point can be divided into three regions Variation of the pH in the acidic compartment affects mainly the distribution of the base and should therefore shift the point into region 1 Variations of the pH in the alkaline compartment affects mainly the distribution of the acid and should shift the point into region 2 Variations of the pH in both intracellular compartments in such a way that the intracellular pH differences remain uninfluenced results in a shift along the dotted line parallel to the line $\Delta pH_a = \Delta pH_b$ representing homogeneous systems If the shift is into region 3 the pH values of the two compartments are varied in opposite direction

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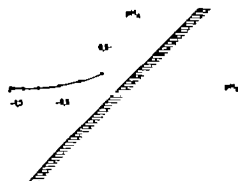


Fig 3: Influence of increasing benzylamine concentrations on the I tracellular pH

Transcellular pH difference calculated from the DMO distribution (ΔpH_a) is plotted versus that calculated from the benzylamine distribution (ΔpH_b) Benzylamine varied from 0 to 10 mM

Table 1: Effect of aerobic glycolysis on pH difference as determined by DMO (ΔpH_a) and benzylamine (ΔpH_b)

	ΔpH_a	ΔpH_b
without glucose	+ 0 05	- 1 42
with 10 mM glucose	- 0 17	1 38

but not the benzylamine distribution (Table 1) That benzylamine does not monitor the cytoplasmic pH is supported by the observation that the addition of 10 mM benzyl amine while increasing the pH of the acid compartment by as much as 1 pH unit has no effect on the membrane potential as monitored by the distribution of the lipophi lic cation tetraphenylphosphonium (TPP^+) (results not shown) Otherwise the neutra lization of the cytoplasmic H^+ ions would lead to a passiv uptake of protons partially depolarizing the cell

The most appropriate explanation of the results is that in respiring cells the cyto-plasmic pH is nearly the same as the extracellular pH that the cytoplasma membrane is impermeable to H^+ ions and that there must be an active mecha ism for the extru-sion of H^+ ions out of the cell That the cytoplasmic pH is only slightly different from the extracell lar pH was recently shown by ^{31}P-NMR of different glycolytic interme diates I the Ehrlich cell (9)

An experimental test of the assumption that the cytoplasma membrane is not pas sively permeable to H^+ ions is shown in Fig 6 To vary the membrane potential cells were incubated in buffers of different K^+ content in the presence of valinomycin One sees no influence f the varied membrane potential on the intracellular pH Only after the addition of the proton conductor FCCP the pH readjusts to the membrane po-tential That mainly the pH of the basi compartment is influenced by changes of the membrane potential adds further support to the assumption that DMO monitors the cytoplasmic compartment

Summarizing the results:

1 There exist compartments of very different pH inside the Ehrlich cell
2 There is evidence that i respiring cells the pH of the cytoplasm differs only slight ly from the extracellular pH
3 The cell lar membrane is impermeable to passive proton movement
4 As a consequence one has to postulate a mechanism for translocating protons against the l lectrochemical potential gradient

Acknowlegments: The authors wish to thank Ms C Herschel for valuable technical assistance The work has been supported by Grant No He 102/14 from the Deutsche Forschungsgemeinschaft

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Appendix

Cells with N intracellular compartments (relative sizes: v_1 v_n v_N; $\Sigma v_n = 1$) of different H^+ ion concentrations (H_1^+ H_n^+ H_N^+) are incubated in a medium with a H^+ ion concentration H_e^+ To determine the intracellular pH (pH_i) the distribution ratios of weak electrolytes are used

1 If a weak acid AH is uncharged and permeates, while the conjugated base A^- does not the intracellular pH is calculated from the relation

$$(pH_i) = pH_e + \log R_a$$

The distribution ratio R_a of the base A is calculated from the distribution ratio $\tilde{R}_a$ of the total concentrations (AH + A) between cell and medium At equilibrium for the acid AH the distribution ratio $\tilde{R}_a$

$$\tilde{R}_a = \frac{\sum v_m (AH_m + A_m)}{AH_e + A_e^-} = \frac{1 + K \sum v_m / H_m^+}{1 + K/H_e^+}$$

and the distribution ratio R_a of the base A^-

$$R_a = \frac{\sum v_m A_m}{A_e^-} = (\tilde{R}_a - 1) H_e^+/K + \tilde{R}_a = H_e^+ \sum v_m / H_m^+$$

2 If a weak base B is uncharged and permeates while the conjugated acid BH^+ does not the intracellular pH is calculated from the relation

$$(pH_i)_b = pH_e \quad \log R_b$$

The distribution ratio R_b of the acid BH^+ is calculated from the distribution ratio $\tilde{R}_b$ of the total concentrations $(B + BH^+)$ between cell and medium At equilibrium for the base B the distribution ratio $\tilde{R}_b$

$$\tilde{R}_b = \frac{\sum v_n (B + BH_n^+)}{B_e + BH_e^+} = \frac{K + \sum v_n H_n^+}{K + H^+}$$

and the distribution ratio R_b of the acid BH^+

$$R_b = \frac{\sum v_n BH_n^+}{BH^+} = (\tilde{R}_b \quad 1) K/H_e^+ + \tilde{R}_b = (\sum v \quad H_n^+)/H^+$$

Since the product of the two distribution ratios

$$R_a R_b = \left(\sum v_m / H_m^+\right)\left(\sum v_n H_n^+\right)$$

$$= \frac{1}{2}\sum\sum v_m v_n \left(H_m^+/H_n^+ + H_n^+/H_m^+\right)$$

$$= 1 + \frac{1}{2}\sum\sum v_m v_n \left(\sqrt{H_m^+/H_n^+} - \sqrt{H_n^+/H_m^+}\right)^2 \geq 1$$

the difference of the pH values as determined by the two methods

$$(pH_i)_a \quad (pH_i)_b = \log(R_a R_b) \geq 0$$

q e d

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Transepithelial Permeability in the Rabbit Pancreas

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Received 21 September 1977

ABSTRACT: In the is lated rabbit pancrea there i transepitheli l pathway which i permeabl f cations and small neutral mol cul s Using a number of neutral radioactive molecules with in easing molecul size it has been hown that th larger the molec l th mor difficult is it permeation Stimulant of th enzyme ecretion p ocess increas th permeability f this pathway Thi eff t is relatively the l rgest for th di sc harides sucrose and la tos Compari on of these sult with determinations f extra llula spac suggests that this trans pithelial pathway i parac llular

Introduction

The pancreas secretes a fluid which i isotonic with the plasma and ontains the digestive enzymes produced by thi o gan Calcium ions re thought t mediat the ff t f pancreozymin and acetylcholine on the secretion f dig stive enzymes Hence an analysi f the calcium content f the ecreted fluid might be f importance fo our unde tanding f th timulus- ecr tion proc s in this rgan V rious uthor found linear corr l tion between the concentr tions of calcium nd protein in the ecreted fluid but ther is le prot in-independent cal ium f tion (1-4) The l tter fra tion amounts in the isolated rabbit pancrea t cal ium concentration f bout 1 mM which is 30-40% f that in the bathing medium (5 6) We lso found that thi prot in-independent cal ium flux is dependent on the concent tion f cal ium in the bathing medium suggesting that it f llows a parac llul r transepithelial pathway When we lso tudied the magnesium concentration in the ecr ted fluid we found that th behaviou f this ion i t least qualit tively simil to that f cal ium (6)

In expe iment where ^{45}Ca ^{28}Mg was added t th bathing medium f the i l ted bbit panc ea the isotope equilibrat in bout 30 min

with the protein-independent part of the calcium and magnesium fluxes in th secr ted fluid (5 6) When ^{3}H-mannitol was added to the bathing medium this compound appears lso in 30 min in the secr ted fluid This suggests that the tr nsepithelial pathway is at least permeable for divalent cations and mannitol (5)

A second effect of thi transepithelial pathway which we observed is that after stimulation of th enzyme secretion with carbachol th concentrations of ^{45}Ca ^{28}Mg and ^{3}H-mannitol in th secreted fluid further increas reaching a peak after about 40 min which is 20 min after the appearanc of the enzyme peak in the secr ted fluid After the ppearance of the peak the concentr tions decrease again but in most cases the original level is not reached The reason for this transient increase in permeability and its physiological significance are not known

We therefore decided to investig t the properties of this transepithelial pathway in more detail by tudying the permeability of a eries of compounds with increasing molecular weight in the isolated rabbit pancreas

Mat ri l and Method

Carbachol is purchased from ACF Chemiefarma Maarden the Netherlands; cerulitide and bombesin are donated by Dr R de Ca tiglione (F rmitalia Milan Italy) ^{14}C-Urea (sp act 60 mCi/mmol) ^{14}C-gly erol (spec act 38 mCi/mmol) ^{14}C-erythritol (spec act 2 3 µCi/mmol) ^{14}C-mannitol (pec act 60 mCi/mmol) ^{14}C-Lactose (spec act 60 mCi/mmol) ^{14}C-sucr (spec act 5 1 mCi/mmol) and ^{3}H-inulin (spec act 900 mCi/mmol) are btained from The Radiochemical Centre Amersham U K

The preparation of the isolated rabbit pancreas the incubation medium and the fr ction collection have been described previously (5) The radioactive compounds are added to the bathing medium in 2 mM concentr tion The radioactivity in the secreted fluid is expressed s the percentag of the concentration in th bathing medium In the experiment with cold sucros the concentrati n in the bathing medium is 5 mM nd th sucro e content in the s creted fl id is analy ed in 15 inst ad of 5 min fraction

The amount f secreted fluid i det rmined by weighing Prot in is determined according t Lowry et al (7) on ml roscale with bovine serum albumin (Beh ingwerke) erving standard The concent ation of the radioactive compounds in th ecret d fluid i determined by adding 20-50 µl sampl s of secreted fl id and b thing medium t Bray s sol tion

(10 ml) and counting th mixture by liquid intillation spectroscopy In the experiments with cold ucros this compound is analysed by measuring the amount of NADPH formed fter hydrolysi f th sucro by β fructo- idase f llowed by oxidation of the sulting glucos by mixtur of hexokinas and gluc se-6-phosphate dehydrogenase in the pre ence f ATP and NADP (8) The ugars in the ecr ted fluid ar analysed by thin layer chromatography on Chromarsheet 500 (Mallinckrodt) using chl rof rm- methanol-acati cid 70:30:15 as developing solvent

Det rmination f the extra llular spac in rabbit pancreas lices is s entially as d scribed f r mammary gland lices (9) with th diff renc that besides ^{3}H-inulin a ries f oth radioactiv compounds is sed

Results and Discus ion

The is lated rabbit panc eas sec tes at nearly constant te during th maximal 4 hour f each experiment (fig 1 3) When after 60 90 min f incubation carb chol i added to th bathing medium ther is in most expe iments a bri f eduction in the fluid ecretion rat th reason f r which is unknown Addition f carbachol always results in a marked increase in enzyme secr tion, sulting in peak aft 15-20 min (fig 1 3) The time lapsing between addition f the timulant nd the appearance f the enzyme peak efle ts mainly the transport time f th enzyme from the point f rigin to th end f th pancreati duct

Fig 1 hows an experiment in which 2 mM ^{14}C-urea i added to the bathing medium at th beginning f th experiment After about 40 min the concentration in the ecreted fluid has equilib ted with that f the bathing medium Afte stimul tion with 10 ^{5}M carbachol no furthe change in the

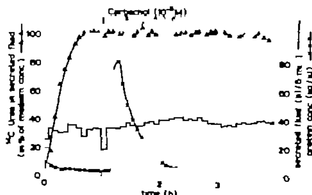


Fig 1

Permeability f ^{14}C-urea in th isolated abbit panc eas At t=0 2 mM ^{14}C-urea and t t=1h 10^{-5}M carba hol is added to the bathing medium Rat of fluid secr tion (—) and concentrations f protei (-x-) and ^{14}C-urea (-Δ-) in the e cr ted fl id re measu ed

[illegible] is obtained
[illegible]
[illegible] trol is [illegible]
[illegible] in the [illegible]
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[illegible] extraction
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concentration however does not exceed 5% f that in the b thing medium. In this case timul tion of th enzyme secretion with ca bachol causes a very marked in reas in the concentrations f these compounds in the ecr ted fluid A peak is reached about 35 min aft addition f the timolant (i about 20 min after the appea anc of th enzyme peak) Thereafter th concentration f the radioactive compound in the secr ted fluid decreases gain but it does not return t the riginal lev l

Table I hows that th magnitud f the ca bachol effect on the concentr tion of the di accharides in th e cr ted fluid aft r timulation depends on the carbachol oncentration Th small effect bs rved with 10^{-7}M carbachol may not be ignificant inc in the absence f stimulation ther i lso an increas in th concentr tion of the radioactiv compound

When the d capeptide arulitide (caerul in 6 10^{-9}M) and the t tradeca peptid bombesin (4 10^{-9}M) re used to stimul t th enzyme ecr tion th effects on the appearance f sucro in th secr ted fluid re the same as with 10^{-5}M carbachol

T mak sur that in the experiments with ^{14}C-sucros the measured radioactivity can be attributed t the intact disaccharid two additional

TABLE I

EFFECT OF CARBACHOL ON CONCENTRATIONS OF SUCROSE LACTOSE AND PROTEIN IN THE SECRETED FLUID OF THE ISOLATED RABBIT PANCREAS

Carbachol concentr tion	SUCROSE before timul tion	SUCROSE aft timul tion	PROTEIN ti	
10^{-5}M	3 4 ± 0 2	21 4 ± 2 7	15 0 2 6	5
10^{-6}M	4 8 ± 1 0	7 4 ± 0 9	6 2 ± 1 9	4
10^{-7}M	5 3 0 2	6 3 ± 0 7	1 1 ± 0 1	2
no timula t	4 5 0 1	5 8 ± 0 1	1 0 ± 0 1	2
	LACTOSE		PROTEIN	
10^{-5}M	5 1 ± 0 5	30 9 ± 1 0	11 9 ± 3 7	3
10^{-6}M	5 1 ± 0 2	9 7 ± 0 6	8 0 ± 0 5	2

Values for sucroe and lactose represent th percentag of the concen tr tion f th label in th bathing medium (2 mM) "Befor timul tion" f rs to the value during the 30-mi pe iod bef addition of car bachol ft timulation t the av g value 35-45 mi fter addi tion f the timolant V lues f prot in ec tion r p se t ti between the ecreted amount of prot in in the 30-mi pe iod aft addition f carbachol t that bef addition V lues ar with S E M ; n represent the numbe f expe iment

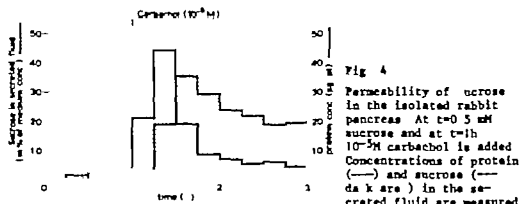


Fig 4
Permeability of ucrose in the isolated rabbit pancreas At t=0 5 mM sucrose and at t=1h 10^{-5}M carbachol is added Concentrations of protein (——) and sucrose (——da k are) in the secreted fluid are measured

experiment have been performed Fir t analysis of the sugars in the secreted fluid by thin layer chromatography shows that all radioactivity is in the sucrose spot Secondly non-radioactive sucros instead of ^{14}C-sucrose is add d to the bathing medium and the sucrose content in the secreted fluid is analyzed enzymatically (fig 4) Essentially the same obs rvation i mad as with ^{14}C-sucrose confirming the conclusion that upon stimulation the pancr atic epithelial tissue becomes permeable for intact disaccharides

The q estion now a ises whether the transepitheli l pathway leads through the cells r through the so-called tight junctions between the c lls In the fir t c se one would have to assume that these radioactive compounds penetrate into the c lls in particul r after stimulation with carb chol when they are incubated with rabbit pancreas slices

TABLE II

PERCENTAGE OF TOTAL TISSUE WATER REACHED BY RADIOACTIVE MARKERS IN RABBIT PANCREATIC SLICES

Compound used	unstimul ted	stimulated
urea	95 ± 4 9 (4)	
glycerol	137 ± 7 6 (4)	
erythritol	81 ± 3 6 (5)	
mannitol	48 ± 2 5 (3)	
lactos	40 ± 1 3 (5)	42 ± 1 7 (4)
sucros	39 ± 1 6 (6)	40 ± 1 4 (4)
inulin	33 ± 1 3 (10)	-

Values ar given with S E M nd between parentheses the number of det rminations Ca bachol (10^{-5}M) ha been d timulant and is present during th 90-min inc bation period

Thi has been studied by d termining the space occupied by thes radio-a tive compounds which would then exceed the extrac llular space i e th pac occupied by a non-penetrabl compound like inulin Tabl II shows that th extracellular spac measured with ^{14}C-inulin i bo t 33% The spaces occupied by sucro lactose and mannit l ar not or only slightly larger than that f inulin Th tendency towards omewhat large values may be due to the fact that part of the extrac llul r space can be better reached by small mol cules than by inulin Moreover the finding that the ucrose and lacto spaces d not increase upon stimul tion with carbachol trongly suggests that thes compound do not enter th cells On the other hand urea glycerol and t smaller extent erythritol appear to penetrat into the cells The high value f glycerol may be du to metabolism f thi ompound in the cells

Thes experiment sugge t that the transepith lial pathway i para-cellular rout through the tight junctions This finding raises eve l questions First wher is thi parac llular route located: between duct-ular c lls r in the cini between inar r acina /centro-acina ll ? Secondly what is th mechanism of the increas in permeability aft pplication of timulants f the enzyme secretion and why is the espons low? Po sibly there i chang in the morphology f the tight junc-tions between the acinar cell as a r sult f the restoration f the apical memb ane urfac f thes lls after timul tion f the enzyme ecretion Thirdly what i the physi logical function f thi phenomenon if there is any? Whil it ould be an rtifact occurring only in the isol ted r bbit pancreas it may lso be an important phenomenon taki g plac lso in ther epithelial tissues and playing a rol in the regula tion of the electrolyt compo ition of sec t ry fl ids Further tudi f thi phenomenon a ther f in order

The excellent technical as i tanc f Mis A Jakob is gr tefully cknowledged

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Ca^{++} Control of Electrolyte Permeability Effect on Na^{+} Fluxes in Isolated Membrane Vesicles from the Cat Pancreas

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ABSTRACT: Th influence f Ca^{++} on $^{22}NaCl$ fflux from isol ted membran vesicles f cat pancrea has been me sured The data presented show that NaCl efflux is increased in th sequenc : Ca^{++} present t the vesi l out ide only C ++ present at both ides Ca^{++} pr ent at the insid only Increa ing Ca^{++} concentration ($10^{-6}M$-$10^{-3}M$) applied t th v i l insid t a constant Ca concentr tion t the vesicle ut-sid ($10^{-4}M$) howed increased rate of ^{22}N Cl efflux. The dat support the hypothesi that th NaCl permeability in pancreatic acinar ll membranes is controlled by Ca^{++}

INTRODUCTION

During the last years a l rge body of evidenc has been obtained indicating that in the action f sev ral hormones c lcium has the function f an intracell lar econd mes enger In secr tory tissu whe hormones timulat the secretion f lectr lyt s and fluid the mediat r f ecr tagogues mo t lik ly influence ither activ transport or the permeability f the cell membran f those lectrolytes involved in secret ry proces s Conc rning the exocrine pancreas indirect evidenc sugg t that Ca^{++} i th mediato f the ffect of ac tylcholine and pancreozymi on both enzyme NaCl and fluid secr tion Electrophysiological tudi have hown that depolarization induced by secr tagogues can b expl ined as due t an increa ed permeability f th contr luminal c ll membra t Na and K (1 2) Further evidence for Ca^{++} mediated increa in Na permeability came from the b erv tion that

Ca^{++}-dependent depolarization could be induced by the Ca^{++} ionophore A23187 In the absence of Na this d polarization was abolished suggesting that depolarization is due to an increased influx of Na^{+} resulting from a Ca^{++}-mediated increase in Na^{+} permeability (3) In addition the finding that intrac llular Ca^{++} pplication results in membrane depolarization similar to that evoked by acetylcholin suggests that th ac tylcholine effect i caused by a ris of intracellular free Ca^{++} which in turn influences the Na^{+} permeability (4)

In order to get a direct proof if intracellular Ca^{++} increases the Na permeability of pancreatic cell membr nes we have studied the influence of Ca^{+} on Na^{+} fluxes in isol ted membrane vesicles from cat pancreas

Our d ta show that in isolated pancreatic membranes Ca^{+} applied at the vesicle outside decreas s but Ca^{++} at the vesicle inside increases th NaCl permeability These observations th refore support the hypoth sis that Ca^{++} acts as a second messeng r ex rting direct effect on the membran permeability for thos electrolyt which re finally secreted from the acinar c lls

METHODS AND RESULTS

Pl sma membranes were prepared by zonal centrifugation on an exponential ficoll-sucros gradient by a previously d scribed method (5) Briefly it involves mincing and homogenization of t pancreas in an ice-cold sucrose buffer by means of a hand-operated glass-t flon homogenizer and centrifugation of the material for 3 5 hrs at 100 000 g through a continuous exponential ficoll-sucrose gradient f rmed in a Beckmann Ti 14 zonal r tor Four protein bands wer recovered from the gradient which r f rred to plasma membranes endopl smic r ticulum mitochondria and zymogen granules as d termi ed by lectron microscopy and the presenc of typical marker enzymes for the respectiv organelles (5)

The plasma membrane fra tion enriched in (Na K)-stimulated Mg^{++} dependent ATPa e hormone-stimul t d adenylate cycl se and 5 -nucleoti dase was used for ^{22}Na-efflux experiments The tightnes of these vesi cles was shown by their respons to L alanine uptake t diff rent os

molarities indicating a corr lation b tween intravesicular spac and the amount f L lanine taken up (6)

To t st the influenc of Ca^{++} on Na fluxes in thes membrane vesi- les they were preincubated for 20 hrs in 200 mM mannitol + 10 mM ^{22}NaCl pH 7 at 4^{o}C If the ffect of Ca^{++} from the in ide of the vesi les r from both ides was to be t ted th preincubation medium contained Ca^{++} at different concentration Ca^{++} concentr tions of 10^{-5}M and lower wer adjusted by a Ca^{++}-EGTA buff After pr incubation an aliquot of this membran s spen ion wa transferred t the incubat- ion medium which contained 200 mM mannit l with or without Ca^{++} but without Na Dilution was 50-f ld At diffe ent period an liquot of this incubation medium was filtered through Millipor filter (HAWP 02500) and washed with icec ld 200 mM mannit l solution Filter were dissolved in 10 ml Bray solution and counted in Packa d Tricarb sci till tion counter

As shown in Fig 1 10^{-4}M Ca^{++} concentr tion pplied from the in- sid f the ve icles increa ed ^{22}NaCl efflux as ompared t that when Ca^{++} was p esent at both id s whereas Ca^{++} being pre ent t the out- side only decreased ^{22}NaCl efflux Afte 15 hr ll ^{22}Na efflux data reached the same equilibrium value which was 2% f th amount f ^{22}NaCl remaining in the vesicl s a compa ed to the o iginal value t 0 time When mimicking the itu tion in vivo i a high extracellular and a low intrac llular Ca^{++} concent tion the ffect of low Ca^{++} con- centrations at the inside f membran ve icl s is even mor pronounced as shown in Fig 2 At Ca^{++} concentr tion t the inside f the ve i- cles as low s 10^{-6}M the fl x rat already increased t a value twi that btained in th absence f intr vesic la Ca^{++} At the highest in- side Ca^{++} concentr tion t ted (10^{-3}M) the rat f ^{22}N efflux was even ix times higher than without Ca^{++} t th vesicl insid

When ve icl were pr incubated with the Ca^{++} ionophore A23187 the ffect f both Ca^{++} at the out id only or Ca^{++} t the vesi l insid only wa aboli hed (data not hown) The effect f Ca^{++} b erved on NaCl fflux wa not comparabl to that on ith RbCl o CsCl efflux In both cas Ca at 10^{-4}M had small r ffect (about 60% l s) when pplied from the outsid but had no ffect when applied from the insid (dat not hown) When th cations were t sted on thei

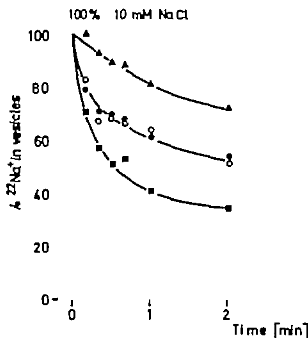


Fig 1 Effect of Ca^{++} on $^{22}NaCl$ efflux plasma membran vesicle of cat pancrea o - o without Ca^{++}; - with Ca^{++} at both sid s of the vesicl (1×10^{-4} M); ▲ - ▲ with Ca^{++} at the vesicle outside (1×10^{-4} M); ■ - ■ with Ca^{++} t the v sicle inside (1×10^{-4} M)

effects on $^{22}NaCl$ efflux the results showed that Sr La^{+++} and Co^{++} had a comparabl eff t on $^{22}NaCl$ fflux as Ca^{++} Mn^{++} and Ba^{++} had no ffect when applied from the insid but inhibited $^{22}NaCl$ efflux when applied from the outsid Th effect of Mg^{++} was similar to that of Ca^{++} although smaller

To check whether the bs rved effects of Ca^{++} were due to a chang of Na^+ or Cl^- permeability and not to int rferenc of Ca^{++} with Na binding sites it wa ec ssary to stablish that at 10 mM Na the concentration used i our experiment Na fluxes and not displ cement of Na^+ by Ca^{++} were me sured A Sc tchard plot of the amount f Na taken up at varying initial Na oncentrations showed that bove 2 5 mM a

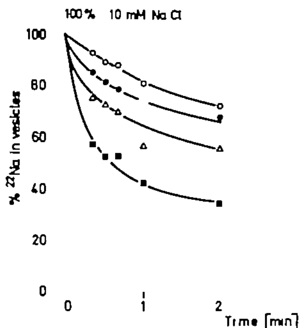


Fig 2 ^{22}Na fflux f om membrane vesi les t different Ca^{++} concentrations t the insid but at constant Ca^{++} concentration (10^{-4}M) t the vesicl outsid - without Ca;; with 10^{-6}M Ca; Δ Δ with 10^{-4}M C ;; ■ ■ with 10^{-3}M Ca;

ero lope wa bserved as would be xpected f uptake int a non-saturating intrave icular volume Below 2 5 mM the lope f the line sugg st that some binding may occur which accounted for bout 10% f the total uptake So th concl ion i j tified that t 10 mM NaCl t least initially binding an b negl ted and the observed ffect ar due t the Ca^{++} control f NaCl permeability

DISCUSSION

Th dat pre ented support the hypothe i that N Cl perme bility i acina ll membran i ontrolled by Ca^{++} In ontr t t data b-

tained from intact cells (3 4) the pre ent results indicate a direct effect of Ca^{++} on the cell membrane whose effective concentration could be asse sed by using varying Ca^{++} concentrations We have no proof that the effect observed ar related to acinar cells howev r sinc the contribution of membran s of th r origin than from acinar cells in our preparation using th ficoll-sucros gradient is negligible (7) it is very likely that the bserved effect i l cated in these cells We als cannot diff rentiate between luminal and baso-lat ral membranes in this fraction and therefore the exact location of the Ca^{++} effects on NaCl permeability is not possible from our data However in analogy to th eff ts in intact tissue using the Ca^{++} ionophore A23187 (3) or intracellular Ca^{+} injections (4) localization at the basal c ll side is very lik ly

Anoth r qu tion conc rns the orientation of membrane vesicles A comparison t the ituation in intact cells would only make sens if the membranes are orientated outside-out i e the same as in vivo We have some evidence that most of the vesicles are orientated outside-out ince the Na^{+} K^{+} stimulated Mg^{++}-d pendent ATP se is activated if the vesicles ar destroyed by det rgents indicating that ATP-binding sites in most of the v sicles ar located at the cytoplasmic sid of the membrane

Unfortunately we have not observed any hormon ffect on Na efflux yet which in vivo should b mediated by cal ium It is suggested by *electrophy iol gical experiments s well as by direct uptak studies of* ^{45}Ca into isol ted acinar cells that both ac tyl holine and pancreozymin displ e Ca^{++} from the inside f the cell membran (8) and increas Ca^{++} influx into the cells to maintain su tained enzyme ecretion (9)

The rise of fr Ca in the cell would increas the NaCl perme bility and th refor NaCl influx into the cell thereby d polarizing the cell From our dat we cannot distinguish between incre s in ither Na or Cl perme bility or of both El ctrophysiologic l data f Petersen and his colleague (10) howev r suggest that ac tylcholine incr as both Na and Cl perme bility of th acinar ell membrane and to a smaller extent that of K too Ou d t on $^{22}NaCl$ fflux agr e with these bser-vations and comparing ^{86}Rb w th K lso suggest a diff rence in membrane permeability f r both N and K Th dat d scribed here have some simi

l ities with bservations in the tissues wher a chang of th mem- b ane permeability f r K (11) r for Cl (12) induced by Ca^{++} has been observed In th present Symposium th b rvation f Michelang li (13) i f special interest that Ca^{++} inc eas K^+ influx into membrane vesi- les which in turn would stimul t K H^+ exchange pre ent in g tric mucosa (14) Thu Ca^{++} ould be con idered as the intra ellular t igg f HCl secr tion in gastri mucosa as well as of NaCl ecr tion in pan- creatic acinar 11

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H^+ Sugar Cotransport in Bacteria

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ABSTRACT Evidence was reviewed for the hypothesis of Mitchell that there is obligatory coupling between proton movement and sugar movement across the plasma membrane of microorganisms Data were presented to support this hypothesis for the case of proton thiomethylgalactoside cotransport in Escherichia coli and Streptococcus lactis Sugar addition to energy depleted cells resulted in proton entry proton addition to such cells resulted in sugar accumulation Quantitative experiments were reported which are consistent with the stoichiometry of one proton for one sugar

In 1961 Peter Mitchell (1) proposed that energy derived from biological oxidation could be stored in the form of an electrochemical potential difference of protons ($\Delta\mu_{H^+}$) across a membrane Since that time it has been clearly shown that such $\Delta\mu_H$ (protonmotive force) may be observed across many types of membranes Proton pumps include a) respiratory chain of mitochondria and bacteria b) light sensitive bacteriorhodopsin c) light sensitive mechanisms of chloroplasts and d) membrane bound Ca^{++} Mg^{++} dependent ATPase of mitochondrial and bacterial membranes The energy stored in the ion gradient is utilized by the cell for several membrane related processes one of which is proton substrate cotransport

In 1963 Mitchell suggested (2) that there is obligatory coupling between proton and lactose entry into Escherichia coli According to this view energy for the accumulation of sugar comes from the electrochemical potential difference of protons across the membrane As protons enter the cell with lactose they reduce the $\Delta\mu_H$ across the membrane This stimulates the respiratory chain which pumps out the H restoring the initial $\Delta\mu_H$ This mechanism was analogous to that previously suggested by Crane

(3) for Na^+ glucose cotransport by the mammalian small intestine Evidence supporting the Mitchell proposal has been accumulating in recent years (see reviews by Harold (4) and Hamilton (5))

Two general types of evidence tend to support this hypothesis The first involves the demonstration of proton movement against an electrochemical potential difference in response to the addition of lactose to energy depleted cells (Fig 1) The second involves the converse experiment namely sugar accumulation induced by artificially induced $\Delta\mu_{H^+}$ The first of these experiments was carried out by West (6 7) and West and Mitchell (8) The addition of thiomethylgalactoside (TMG) to energy depleted E coli resulted in proton entry Fig 2 shows an experiment of West in which the addition of TMG to anaerobic E coli suspended in un buffered solution resulted in an alkalinization of the external medium. Parallel experiments (7) showed that addition of TMG resulted in acidification of the cytoplasmic compartment Thus TMG entry drove protons into the cell against a pH gradient

The converse experiment may also be carried out in E coli An artificially induced inwardly directed protonmotive force will drive sugar entry into the cell against a concentration gradient (Fig 3) The protonmotive force may be produced either by a membrane potential (inside negative) or a pH gradient (outside acid) or both Specific examples will illustrate these experimental approaches A membrane potential was artificially produced by first incubating energy depleted E coli in valinomycin to make them permeable to K and then diluting the cells into a K free medium (9) Fig 4 shows that the membrane potential resulting from K^+ exit drove the TMG entry and accumulation Dilution of cells into K^+-containing solution

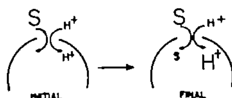


Fig 1 A prediction from the co transport hypothesis Addition of substrate (S) to energy depleted cells drives H^+ against a gradient

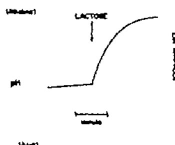


Fig 2 Lactose addition to energy depleted cells drives proton entry The pH is measured in an anaerobic suspension of E coli Redrawn from West (6)

did not show accumulation of TMG

An alternative method of producing a protonmotive force is with an artificial pH gradient Energy-depleted cells were exposed to radioactive sugar at pH 8 (Fig 5) The intracellular TMG concentration was only very slightly higher than that in the medium. When sufficient HCl was added to reduce the pH to 5 9 a marked TMG accumulation resulted

Quantitative experiments

West (6) was the first to correlate proton movement and sugar movement in energy depleted _E. coli._ He found one proton moved per sugar molecule

A second approach was to correlate the total protonmotive force with the chemical potential of TMG If there were obligatory coupling between H^+ entry and sugar entry (with a 1 1 ratio) then the electrochemical potential of H should be in equilibrium with the chemical potential of the sugar according to the following relationships:

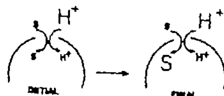


Fig 3 A prediction from the cotransport hypothesis Imposing an inwardly directed $\Delta\bar{\mu}_H$ drives S accumulation

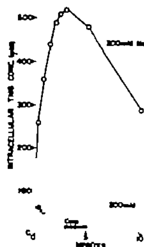


Fig 4 Membrane potential drives TMG accumulation Energy depleted cells of ML 308 were preincubated in the presence of valinomycin and 200 mM K^+ and then diluted 50 fold into 200 mM Na^+ or K^+ phosphate buffer

a) $\Delta\mu_{H^+} = \Delta\Psi \quad Z\,\Delta pH$

where $\Delta\Psi$ is the membrane potential and Z is equal to 2 3 RT/F or a value of 59 at 25°

b) $\Delta\mu_{TMG} \quad Z \log \frac{TMG(IN)}{TMG(OUT)}$

c) At equilibrium $\Delta\mu_{H^+} = \Delta\mu_{TMG}$

To obtain experimental values for $\Delta\mu_{H^+}$ both membrane potential and pH gradient must be estimated. The pH gradient was estimated indirectly by the distribution ratio of certain weak acids such as dimethyloxazolidine dione (DMO) or acetylsalicylic acid This method takes advantage of the fact that the free acid readily crosses the membrane while the ionized species does not Knowing the ratio of intracellular to extracellular



Fig 5 pH gradient drives TMG accumulation Energy depleted cells of ML 308 were incubated in the presence of radioactive TMG At the arrow HCl was added

acid the pK_a and extracellular pH application of the Henderson-Hasselbalch equation will give the intracellular pH

The membrane potential has been estimated by three general methods fluorescent dyes (10) lipid soluble cations (11) and K^+ distribution in the presence of valinomycin (see general discussion of methods in reference 12) The latter method was used for the experiments to be described. Valinomycin a K specific ionophore is added and the ratio of the potassium concentrations inside and outside is measured. The Nernst equation is then applied in the following form

$$\Delta\Psi \quad 59 \log \frac{K\ (IN)}{K^+ (OUT)} \quad \text{at } 25^{o}C$$

Experiments with S lactis (13 14) and E coli (9 15) indicate that there is good correlation between $\Delta\mu_{H}+$ and $\Delta\mu_{TMG}$ The experiments reported here represent an extension of the studies with S lactis An improvement in technique was the use of the microfuge instead of the filtration method for the determination of pH The cells were centrifuged through silicone oil causing the contaminating extra cellular fluid to be greatly reduced and thus increasing the accuracy of the intracellular measurement In addition acetylsalicylic acid and benzoic acid have been used as intracellular pH monitors as well as DMO These acids have lower pK values than that of DMO enabling measurements to be made at lower external pH values In several experiments the three weak acids were compared in glucose fermenting cells at pH 7 and the calculated intracellular pH was found to be the same by the three methods

The experiment given in Table 1 shows a time course of $\Delta\mu_{H}$ development and TMG accumulation representative of cells incubated with glucose at pH 7 The cells generate ATP from substrate level phosphorylation in glycolysis ATP activates the membrane bound proton ATPase to extrude protons and develop a protonmotive force As the $\Delta\mu_{H}+$ increases the capacity to accumulate TMG increases

The correlation of $\Delta\mu_{H}$ and $\Delta\mu_{TMG}$ for a series of experiments is shown in Fig 6 Several different experimental conditions were used in this series The incubation medium varied between pH 5 and pH 7 and the three weak acids mentioned above were used in different experiments to monitor the intracellular pH In the absence of fermentable substrate the $\Delta\mu_{H}$ and TMG accumulation are low When arginine is added much higher accumul tion is observed and with glucose the maximum $\Delta\mu_{H}$ is

Time Course of $\Delta\mu_{H}$ and TMG Accumulation

TIME (Min)	$\frac{K_{(IN)}}{K_{(OUT)}}$	$\Delta\Psi$ (mv)	ΔpH	$\Delta\mu_{H}$ (mV)	$\frac{TMG_{(IN)}}{TMG_{(OUT)}}$	$\Delta\mu_{H}^{+}$ (mv)	$\Delta\mu_{TMG}$ (mv)
	7 35	51	0 36	21	5 6	30	44
10	9 7	58	0 50	30	19	88	75
20	11 2	62	0 61	36	32	98	89
30	12 5	65	0 63	37	52	102	101
40	13 7	67	0 67	40	89	107	115

Washed cells of S lactis at a final OD of 1300 were incubated in ^{14}C DMO (0 005mM) ^{3}H inulin glucose (11 mM) TMG (0 003 mM) valinomycin (1 μM), K_2HPO_4 (30 mM) Na_2HPO_4 (50 mM) adjusted to pH 7 Duplicate samples of 1 ml each were removed and placed in a microfuge tube containing 0 5 ml of silicone oil After centrifuging for 1 min supernatant and oil layers were removed and the pellet obtained by cutting across the tip of the plastic tube The cells were digested in 1 M NaOH a sample removed for K^+ determination and the remainder counted in a liquid scintillation counter The intracellular DMO concentration was calculated after correction for extracellular fluid space (inulin space) In a parallel flask cells were incubated in a similar solution except that TMG was radioactive while the inulin and DMO were not Cells were centrifuged in a similar manner and intracellular and extracellular TMG determined

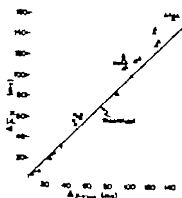


Fig 6 Relationship between $\Delta\mu_H$ and $\Delta\mu_{TMG}$ in *Strep lactis* Experimental conditions similar to those given in Table 1 (whose data is included in the figure) Cells were incubated either with no substrate (circles) arginine (squares) or glucose (triangles) The three triangles with $\Delta\mu_H$ below 80 mv represent experiments in which cells were incubated with glucose for a short period (2 minutes) All other experiments involved incubation periods of from 10 to 40 minutes

observed. There is a reasonably good agreement between the $\Delta\mu_H$ and $\Delta\mu_{TMG}$. This is consistent with the hypothesis that there is an obligatory coupling between proton movement and TMG movement with a stoichiometry of 1 1

Variations in the stoichiometry of protons and substrate have been noted Collins *et al* (16) have grown *E. coli* in a chemostat with alanine as a sole carbon source and as a limiting nutrient While the normal cells show a stoichiometry of 1 proton/alanine uptake two mutants isolated from the chemostat showed 2 protons/alanine and 4 protons/alanine The ability to accumulate alanine would be enhanced at the expense of increased energy per alanine molecule

Another example of altered stoichiometry is given by Ramos and Kaback (15) who showed a change of 1 proton/lactose to 2 protons/lactose by increasing the pH from 6 to 8

An extensive literature now exists (4 5) on proton substrate cotransport in a wide variety of cells Hexose proton cotransport has been demonstrated in *Chlorella vulgaris* (17) *R. gracilis* (18) and *Neurospora crassa* (19) Amino acid-proton cotransport has been studied in yeast (20) and in bacteria (21 23) It therefore appears that the cotransport of protons with various substrates is a fundamental and widespread phenomenon in many microorganisms

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H^+ Transport in Gastric Microsomes Towards a Molecular Model for Secretion

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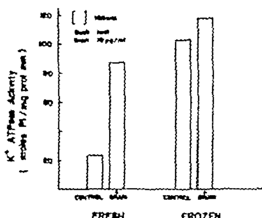
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ABSTRACT H^+ transport and K^+ ATPase activity were measured in gastric microsomes Activities are controlled by the K^+ permeability of the vesicular membrane Ca^{2+} (μM range) binds to the vesicular membrane and increases K^+ permeability H^+ transport and K^+ ATPase are increased by virtue of this effect. A model is proposed for the role of Ca^{2+} as a messenger in mediating stimulus secretion coupling in the intact parietal cell

The role of ATP on the energy source for gastric acid secretion is still controversial The recently found H^+/K^+ ATPase of gastric microsomes (1 2 3) may provide a means of coupling between energy metabolism and the H^+ pump in the intact cell

The rate limiting step for the hydrolisis of ATP and H^+ uptake appears to be the passive permeability of the vesicular membrane to K^+ If this system is responsible for the H^+ traslocation in the intact cell the rate of acid secretion could be then controlled by changes in the K^+ permeability of the vesico tubular and/or apical membrane Thus there could be a coupling between hormonal stimulation and secretion through a change in K^+ permeability Although it is possible to change K^+ permeability and increase ATPase activity by artificial methods (ionophores and freezing thawing for example) one should be able to find a physiological a ent as a means of coupling Calcium ion may play such a role: it has been shown to increase K^+ permeability in red blood cell (4 5) liver cell (6) and nerve membranes (7) Furthermore Ca^{2+} has been shown

Fig 1 K^+ ATPase activity of fresh and frozen micro somal preparations in con trol conditions and in the presence of 10 μg/ml gra micidine



essentially no further increase by the addition of the iono phore These results suggest that the site for K^+ stimulation is inside the vesicle which is normally impermeable to K^+ Similar results have been reported by other authors (2 16)

The dependence of the K^+ ATPase on medium K^+ concentration is shown in Figure 2 The fresh preparation shows a maximal activity (30%) at 100 mM K^+ in a diffusion kinetics pattern Addition of gramicidine or freezing thawing increases the sti mulation of the ATPase by K^+ and shifts the optimal concentra tion from 100 to 5 mM K^+ Furthermore the ascending part of the curve (0 5 mM) shows a saturation kinetics with a K_a of about 0 7 mM for K^+ activation (not shown)

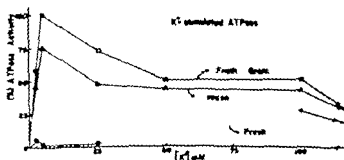


Fig 2 K^+ dependence of K^+ ATPase of fresh and frozen mic osomal preparations with and without 10 n/ml gramicidine

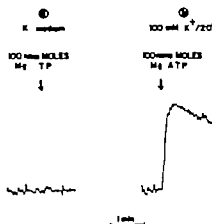


Fig 3 Effect of an ATP pulse on H^+ uptake by fresh vesicles in the absence or presence of ATP

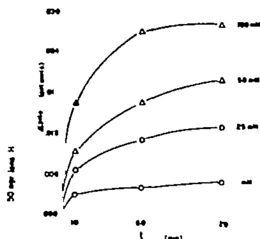


Fig 4 Effect of time of preincubation at different KCl concentrations upon ATP driven H^+ uptake by fresh vesicles

These results indicate that the true optimal concentration is 5 mM in the readily permeable preparation and a gradient force is needed for maximal activation in the permeable (fresh) preparation

The ability of this vesicle to transport H^+ is shown in Figure 3 It can be seen that H^+ transport occurs only in the presence of K^+ Moreover the amount of H^+ uptake driven by ATP hydrolysis is dependent on the amount of K^+ that has permeated into the vesicle at the time of the ATP pulse This can be inferred from the results presented in Figure 4 The dependence on time of preincubation and concentration of KCl of the ATP driven H^+ uptake shown in this figure indicates that diffusion of K^+ into the vesicle is essential for H^+ uptake

If the permeability to KCl increased by freezing thawing the vesicle the time required for equilibration is reduced from 120 minutes to 10 minutes or less as shown in Figure 5 However H^+ uptake is still dependent on concentration indicating that the uptake is dependent on the amount of K^+ existent inside the vesicles to be exchanged by H^+ in the ATP driven process These results are thus in accordance with the H^+/K^+

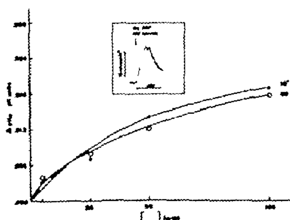


Fig 5 Effect of time of preincubation and KCl concentration on H^+ uptake by freeze thawed vesicles Insert shows time course of H^+ uptake

exchange scheme proposed by other authors (2 7)

The results presented thus far indicate that the activity of the ATPase and the H^+ transport system is in a way controlled by the passive permeability of the vesicular membrane to K^+. A similar mechanism may take place in the intact parietal cell if a means of physiological control of the K^+ permeability is provided. Of the physiological messengers calcium ion appears to be a likely candidate. Thus the effects of Ca^{++} on the K^+ ATPase and H^+ uptake were studied

The effect of 10 µM Ca^{++} on the K^+ ATPase activity is presented in Figure 6. Ca^{++} increases K^+ ATPase activity in fresh

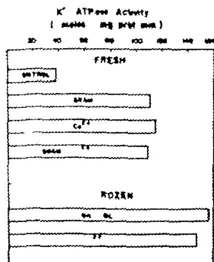


Fig 6 Effect of Ca^{2+} (10 µM) on K^+ ATPase activity of fresh and freeze thawed microsomal vesicles

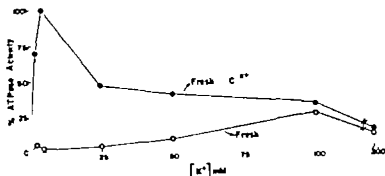


Fig 7 Effect of Ca $^{+}$ on the K^{+} dependence of K^{+}-ATPase of fresh microsomal preparations

preparations in the same way as gramicidine does The effects of Ca^{++} and granicidine are not additive In addition the effect of Ca^{++} is not observable in the readily permeable frozen thawed preparation Figure 7 shows the effect of Ca^{++} *(10 μM) on the K^{+} dependence of the K^{+} ATPase* Ca^{++} shifts the optimal K^{+} concentration for maximal activation of the ATPase from 100 mM in the fresh preparation to 5 mM in the treated one exactly in the same manner as shown before (see figure 2) for gramicidine or freezing thawing Moreover the ascending part of the curve (0 5 mM K^{+}) shows a saturation kinetics with exactly the same K_a measured for the frozen thawed or gramicidine treated preparation (0 7 mM) These re sults show that the effect of Ca^{2+} is that of increasing K^{+} permeability of the vesicular membrane without any effect whatsoever on the K^{+} ATPase per se This effect of Ca^{2+} on the K^{+} permeability of the vesicles has a saturation kinetics with an apparent K_m of 0 38 μM as shown in figure 8 Experi ments in progress appear to indicate that the nature of the binding is of an electrostatic one to fixed negative sites on the membrane

The effects of Ca^{2+} on the H^{+} transport properties of the vesicles are shown in Figures 9 and 10 Addition of Ca^{2+} is able to increase H^{+} uptake by microsomal vesicles (Fig 9) In this experiment K^{+} was present for 15 minutes prior to the addition of ATP Fig 10 shows the K^{+} concentration and time of preincubation dependence of the H^{+} uptake in the absence

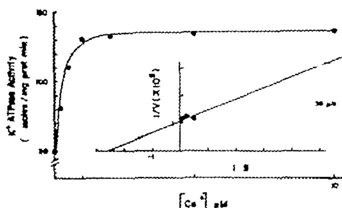


Fig 8 Kinetics of activation of K^+ ATPase by Ca^{2+} in fresh vesicles

and presence of 10 μM Ca^{2+} It can be seen that the magnitude of the uptake is dependent on both K^+ concentration and time of preincubation Ca^{2+} increased the uptake at short times of preincubation without effect on the maximal amount of uptake in the long time equilibrated preparation indicating that

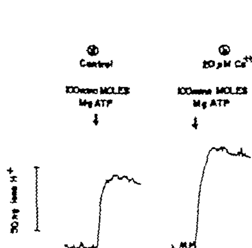


Fig 9 Effect of Ca^{2+} on H^+ uptake by fresh vesicles incubated with K^+

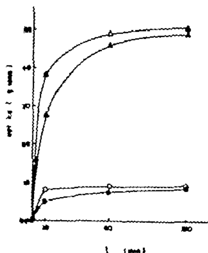


Fig 10 Effect of time of preincubation with 5 mM (circles) or 100 mM K^+ (triangles) in control conditions (filled figures) or in the presence of 10 μM Ca^{2+} (open figures) on H^+ uptake by fresh vesicles

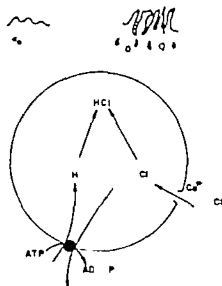


Fig 11 Schematical model depicting the sequence of events involved in H^+ uptake and subsequent HCl formation in isolated microsomal vesicles These events in the intact cell may take place either in closed vesicles such as the tubulovesicular system or at the infoldings of the apical membrane of the secreting parietal cell

this ion has no effect on the transport mechanism per se but rather on the rate of entry of K^+ into the vesicle

The results presented here may be of physiological importance The presence of Ca^{+} in the extracellular bathing fluid is required for the maintenance of acid secretion (8 9 10) The requirement appears to be both intracellular and extracellular (11 12) Furthermore stimulation of secretion is accompanied by increased movement of Ca^{2+} across the cell membrane and/or mobilization of intracellular Ca^{2+} pools (12)

Based on the physiological data and the results presented here we propose a hypothesis for the action of Ca^{2+} in mediating a stimulus secretion coupling in the parietal cell The sub cellular events are pictured in Figure 11 In this model an increase in cell Ca^{+} resulting from hormonal stimulation increases the permeability to K^+ of the tubulo vesicular system and/or the apical membrane of the oxyntic cell In this way K^+ is provided for the H^+/K^+ exchange thus triggering H^+ secretion Therefore calcium is considered to be the final intracellular messenger in the chain of reactions that leads to the production of HCl in the mammalian gastric mucosa

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Transport Parameters of Gastric Vesicles

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ABSTRACT Studies are reported using the effects of osmolarity on gastric vesicle uptake of H^+ indicating that an exchange of H^+ for K^+ or a symport of K^+ with OH is the major mechanism of H^+ ion uptake Using carbocyanine dyes and other techniques the only potentials detected in the vesicles are those due to secondary diffusion potentials Studies with 9 amino-acridine show that there is a discrepancy in the ΔpH by this method as compared to electrode or imidazole uptake measurements The action of SCN^- appears to be inhibition of a forward reaction in the transport rather than increase of leak paths and there is a discrepancy using acridine orange both quantitatively with ΔpH measurements and with respect to SCN action in the that the AO signal is more sensitive to SCN than ΔpH

BASIC OBSERVATIONS In the last few years the study of the enzyme reactions and transport properties of gastric vesicles has become a major frontier in an attempt to understand H^+ transport by the stomach (1 2 3 4 5) The key observations are illustrated in Fig 1 where it is seen that the addition of ATP (at pH 6 1) results in alkalinization of the medium After the ATP has been utilized a combination of a protonophore (tetrachlorsalicylanilide TCS) and a K^+ ionophore (valinomycin) are required to dissipate the formed H^+ gradient Alternatively nigericin a neutral exchange cation ionophore abolishes the gradient Since two conductance pathways must be provided it is tempting to conclude that H^+ uptake by the vesicles is non-electrogenic It must be emphasized

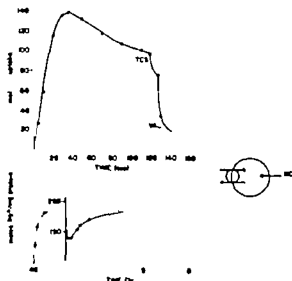


Fig 1 The upper part of the figure shows that with the addi tion of 1 7 X 10^{-5}M ATP H^+ disappears from the medium and both TCS and valino mycin are required to dissipate the gradient The lower part of the figure shows that $^{86}Rb^+$ is extracted from pre-equilibrated vesicles with the addition of ATP

that this assumes the addition of ATP does not change the conductance properties of the vesicles which based on bilayer studies is not correct (6) since ATP increases the K^+ conductance

Also in fig 1 the efflux of $^{86}Rb^+$ is measured from an equilibrium situation with ATP addition, showing that efflux of cation accompanies up take of H^+ (or appearance of OH^-) Again on this basis we may conclude that an H^+ K^+ exchange occurs with the addition of ATP However if the pump were electrogenic driving H^+ into the vesicle and the only conduc tance were K^+ *an efflux of $K^+(Rb^+)$ would be associated with the H^+ uptake* Alternatively the H^+ K^+ countertransport could be a direct result of an H^+ K^+ exchange pump similar in principle to the Na^+ + K^+ ATPase Since it has been shown that Rb^+ efflux is insensitive to protonophores valino-mycin buffer strength or lipid permeable cations the latter conclusion was made more likely (3 5) but not definite Moreover the existence of an H^+ K^+ exchange does not determine whether the exchange is electrogenic or neutral

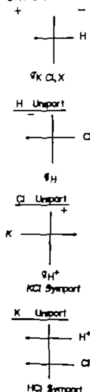
TRANSPORT MODELS Considering that the ion movements are observed with

the sole addition of KCl to the medium the possible mechanisms are classified in fig 2 The simplest type of pump would be an H^+ uniport(electrogenic H^+) with the electrical requirements satisfied by a Cl or K^+ conductance In this situation there is net flux of ions independent of the initial ion content of the vesicles Hence osmotic sensitivity of uptake is always independent of the time of KCl equilibration A Cl uniport associated with a H^+ conductance would give the observed H^+ uptake Osmotic sensitivity would also not be altered by KCl preincubation The above two uniports however would be readily distingusihed by the use

H^+ UPTAKE

UNIPORT/SYMPORT

ANTIPORT



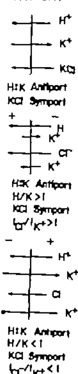


Fig 2 A series of conceptual models illustrating the possible ways in which H^+ uptake occurs due to the ATPase with only KCl added to the medium For brevity OH mechanisms are not illustrated

of ionophores prior to or in the presence of ATP as well as a different sign of the vesicle potential An HCl symport would also provide H^+ uptake and show maintained osmotic sensitivity but would not result in the development of a vesicle potential Not illustrated is coupled H^+ and Cl uniport These would show identical properties to the HCl symport except that with the absence of anion pumping the vesicle would develop an internal positive potential

All of these mechanisms would maintain osmotic sensitivity in spite of internal KCl The next group of mechanisms would show progressive loss of osmotic sensitivity as the vesicles equilibrate with medium K^+

The simplest of the latter is the K^+ uniport mechanism which would generate an interior negative potential and H^+ uptake due to an associated H^+ conductance Antiport processes would be neutral or electrogenic In theory the neutral type would show total loss of osmotic sensitivity as the internal $[K^+]$ reached the maximum $[H^+]$ achievable Electrogenic antiport systems where the H^+/K^+ ratio exceeded unity would only partially lose osmotic sensitivity

OSMOTIC EFFECTS Figure 3 illustrates the finding that with progressive time of incubation with KCl there is a loss of osmotic sensitivity of H^+ uptake The incubation is carried out at 4 C and in previous work it was shown that Rb^+ requires 72 hours incubation to reach equilibrium (5) in the presence of Cl

A second group of mechanisms involves OH /Cl antiport which experimentally is equivalent to H^+Cl symport but is conceptually different However osmotic sensitivity would be maintained with this mechanism

Figure 4 shows the osmotic sensitivity of H^+ uptake where vesicles are placed in KCl solution and ATP is added two minutes later It can be seen that uptake is completely sensitive to vesicle volume and apparently

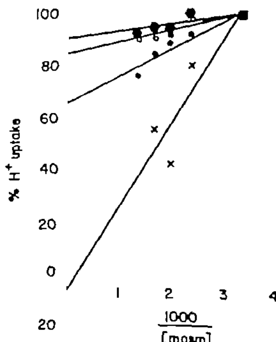


Fig 3 The osmotic sensitivity of H^+ uptake with progressive preincubation times in KCl x—x 5 min •—• 24 hrs o—o 48 hrs ■—■ 72 hrs Medium osmolarity was varied with mannitol

no binding occurs However if one is dealing with a K^+ H^+ antiport the osmotic sensitivity is due to restriction on the K^+ content of the

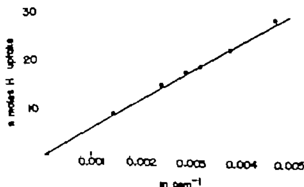


Fig 4 The osmotic sensitivity of H^+ uptake following only 2 min preequilibration with KCl

vesicles With a reversible H^+ uniport trapped buffer would result in a fraction of the uptake being osmotically insensitive since the buffer

content would remain the same although vesicle volume is decreasing However with K^+ H^+ antiport the buffering capacity would be obscured by the effect on K^+ entry The data presented here are consistent with an antiport mechanism that is probably non-electrogenic

Direct measurements of vesicle buffering capacity indicates that it is quite low until about pH 2 However the possible buffering capacity makes it quite difficult to calculate the internal pH reached by the vesicles

QUANTITATION In six experiments 173 nmoles H^+ were taken up by 1 mg vesicle protein This corresponds to a vesicular volume of 2 µl and hence the theoretical pH reached by the vesicle is 1 06 The initial K^+ content of 1 µl is 150 nmoles indicating that only about half of the K^+ is exchanged Assuming that therefore only half of the vesicles transport the pH achieved could be as low as 0 8 close to the maximum attainable by mammalian mucosa

Measurement of the initial stoichiometry of the H^+ uptake showed that the H^+/ATP ratio was close to 4 (3) This rate is thermodynamically impossible for a reasonable value for the ΔG of ATP hydrolysis at a final ΔpH of 5 3 in the vesicle experiments or 6 6 in the intact mucosa To reconcile this one is forced to postulate that either the H^+/ATP ratio is not fixed or that the ΔpH calculated above is much higher than actually the case

VESICLE POTENTIAL To further investigate the role of potentials or to quantitate the ΔpH one is forced into the use of lipid permeable ions We have shown previously that ^{14}C SCN redistributes across the vesicle membrane only in the presence of valinomycin and ATP (3 5) Similar data were obtained for 8-anilino napthalene-1 sulfonate (7) This was interpreted as showing that the only potential generated by the vesicles

was a secondary K^+ diffusion potential in the presence of valinomycin

However our observations were extended to other lipid permeable ions such as the carbocyanine dyes (8) either diethyloxadicarbocyanine (DOCC) or di $SC_{(3)}$ 5 The results with these two dyes were qualitatively identical

In the absence of any ionophore the addition of ATP produces a gradual ($t_{1/2}$ > 60 sec) decline in the absorption of DOCC This change is rapidly reversed by the addition of nigericin and more slowly by lowering external pH On the assumption that nigericin acts as a neutral cation exchange ionophore (i e K^+ for H^+) this indicates that this change of DOCC absorption is due to the presence of ion gradients established by the ATPase rather than due to a potential generated directly by the pump (Fig 5) However the time course of the optical change is much slower than the time course of H^+ uptake (or disappearance from the medium) and would suggest that the ratio of g_{H^+}/g_{K^+} is increasing over the time of change of DOCC absorbance Since DOCC is a lipid permeable cation the vesicle interior is apparently becoming negative Alternatively the vesicles could be developing a Cl conductance

If the membrane conductance for H^+ is drastically increased by the addition of a protonophore TCS then the membrane potential should directly reflect the H^+ gradient developed As shown in Fig 6 the prior addition of TCS followed by the addition of ATP produces a rapid decline in DOCC absorbance This change is reversed by valinomycin triphenylmethylphosphonium ($TPMP^+$) and nigericin indicating that the change is due to an H^+ diffusion potential An alternative way of showing that changes are due to secondary diffusion potentials rather than primary pump potentials is to use in the case of H^+ penetrating buffers such as imidazole As in Fig 6 increasing imidazole inhibits

content would remain the same although vesicle volume is decreasing However with K^+:H^+ antiport the buffering capacity would be obscured by the effect on K^+ entry The data presented here are consistent with an antiport mechanism that is probably non-electrogenic

Direct measurements of vesicle buffering capacity indicates that it is quite low until about pH 2 However the possible buffering capacity makes it quite difficult to calculate the internal pH reached by the vesicles

QUANTITATION In six experiments 173 nmoles H^+ were taken up by 1 mg vesicle protein This corresponds to a vesicular volume of 2 µl and hence the theoretical pH reached by the vesicle is 1 06 The initial K^+ content of 1 µl is 150 nmoles indicating that only about half of the K^+ is exchanged Assuming that therefore only half of the vesicles transport the pH achieved could be as low as 0 8 close to the maximum attainable by mammalian mucosa

Measurement of the initial stoichiometry of the H^+ uptake showed that the H^+/ATP ratio was close to 4 (3) This rate is thermodynamically impossible for a reasonable value for the ΔG of ATP hydrolysis at a final ΔpH of 5 3 in the vesicle experiments or 6 6 in the intact mucosa To reconcile this one is forced to postulate that either the H^+/ATP ratio is not fixed or that the ΔpH calculated above is much higher than actually the case

VESICLE POTENTIAL To further investigate the role of potentials or to quantitate the ΔpH one is forced into the use of lipid permeable ions We have shown previously that ^{14}C SCN redistributes across the vesicle membrane only in the presence of valinomycin and ATP (3 5) Similar data were obtained for 8 anilino-napthalene-1 sulfonate (7) This was interpreted as showing that the only potential generated by the vesicles

was a secondary K^+ diffusion potential in the presence of valinomycin

However our observations were extended to other lipid permeable ions such as the carbocyanine dyes (8) either diethyloxadicarbocyanine (DOCC) or di $SC_{(3)}$-5 The results with these two dyes were qualitatively identical

In the absence of any ionophore the addition of ATP produces a gradual ($t_{1/2} > 60$ sec) decline in the absorption of DOCC This change is rapidly reversed by the addition of nigericin and more slowly by lowering external pH On the assumption that nigericin acts as a neutral cation exchange ionophore (i e K^+ for H^+) this indicates that this change of DOCC absorption is due to the presence of ion gradients established by the ATPase rather than due to a potential generated directly by the pump (Fig 5) However the time course of the optical change is much slower than the time course of H^+ uptake (or disappearance from the medium) and would suggest that the ratio of g_{H^+}/g_{K^+} is increasing over the time of change of DOCC absorbance Since DOCC is a lipid permeable cation the vesicle interior is apparently becoming negative Alternatively the vesicles could be developing a Cl conductance

If the membrane conductance for H^+ is drastically increased by the addition of a protonophore TCS then the membrane potential should directly reflect the H^+ gradient developed As shown in Fig 6 the prior addition of TCS followed by the addition of ATP produces a rapid decline in DOCC absorbance This change is reversed by valinomycin triphenylmethylphosphonium ($TPMP^+$) and nigericin indicating that the change is due to an H^+ diffusion potential An alternative way of showing that changes are due to secondary diffusion potentials rather than primary pump potentials is to use in the case of H^+ penetrating buffers such as imidazole As in Fig 6 increasing imidazole inhibits

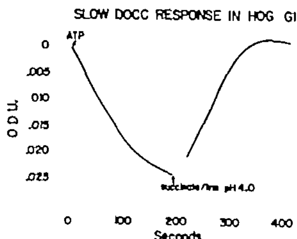


Fig 5 The slow absorbance change induced by ATP with the dye DOCC showing reversal by altering medium pH

the DOCC signal Since imidazole does not inhibit the ATPase the simplest explanation is that with the unprotonated form being freely permeable imidazole reduces the ΔpH across the vesicle membrane Accordingly if the vesicles possessed a reversible electrogenic H^+ pump potential would be maximized as ΔpH minimized since (9)

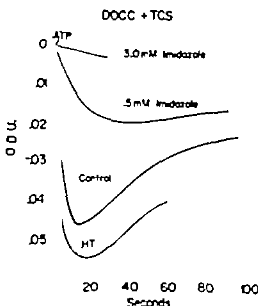


Fig 6 The effect of ATP on DOCC absorbance in the presence of TCS varying concentrations of imidazole and in the presence of hypertonic mannitol

$$\Delta\mu_H \quad \Delta\Psi \quad \frac{RT}{F}\Delta pH$$

Imidazole would maximize the potential if we were dealing with an electrogenic H^+ pump The additional assumption for this conclusion is that the protonated form of imidazole is very much less permeable than the neutral form and therefore imidazole does not act as a good uncoupler of the system. Since imidazole in the absence of TCS does not mimic TCS this assumption is probably correct

The data therefore with ANS as a liphophilic anion and DOCC as a liphophilic cation indicate that although secondary diffusion potentials can be generated by the H K ATPase the enzyme present in hog gastric vesicles does not of itself generate a potential across the vesicle membrane upon energization

It is also possible to use the TCS dependent H^+ diffusion potential to test the effect of osmolarity on the H^+ gradient If it is true that decreasing vesicular volume by osmole addition fixes K^+ content but increases K^+ concentration and thereby increases the $[K^+]$ driving the H^+ exchange then with the quantity of H^+ uptake being constant the H^+ gradient will increase According to this picture increasing the osmolarity will increase the H^+ diffusion potential in the presence of TCS and if this dominates the picture will increase the DOCC signal As shown in Fig 6 this appears to be the case

VESICLE pH Fluorescent dyes or pH indicator dyes may also be used in an attempt to quantitate the intravesicular pH

One of the dyes most frequently used is 9-amino acridine (9 A A] (10) The following equation is used

$$\frac{H^+_i}{H^+_o} \quad \frac{Q}{1-Q} \quad v^1$$

where H^+_i/H^+_o is the H^+ concentration ratio Q is the fractional quench

and v^{-1} is the reciprocal of the fraction of total volume that is intra vesicular space The use of this equation rests on several assumptions such as that the dye which enters the vesicle in response to an H^+ gradient is free and not bound that all the intravesicular dye is quenched; that entry of the dye does not change intravesicular volume and that the dye does not act as an uncoupler

A typical 9 A A response is shown in Fig 7 The action of inhibi tors ionophores and imidazole shows that the 9 A A is responding to a ΔpH Quantitatively this corresponds to a ΔpH of 3 units i e an up take of 1 nmole H^+ μl^{-1} vesicle space This low value could be due to intravesicular buffering capacity or because some of the above assumptions are incorrect In separate experiments it was shown that the signal was only partially osmotically sensitive and the degree of osmotic sensiti vity was critically dependent on 9 A A concentration Accordingly there are binding sites for 9 A A induced by ATP addition At concentra

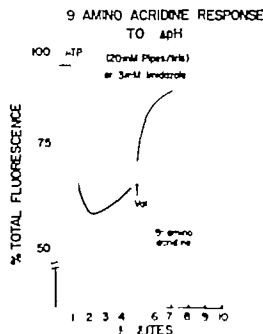


Fig 7 The response of 9 amino acridine fluorescence to ATP addition followed by the addi tion of valinomycin or in the presence of imidazole or strong buffer

tions of 9 A A such as 10 µM where binding makes a less significant con tribution the ΔpH as measured by the pH electrode technique decreases This shows that another problem exists since the efflux of H^+

$$J^{H^+}_{out} \quad k_1[H^+] + k_2[AH^+]$$

where k_1 is the H^+ permeability and k_2 is the permeability of protonated base (eg 9 A A) Even if $k_2 << k_1$ as $[AH^+]$ increases the second term can become significant The loss of pH signal provides evidence there- fore that 9 A A. at concentrations adequate to obscure binding can partially uncouple these vesicles It is therefore questionable whether we can accept that ΔpH of 3 as being true of the situation in the absence of 9 A A

Use of the weak base ^{14}C imidazole and measuring trapping by filtra tion gave data in our best experiments showing uptake of better than 20 nmoles H^+ from calculation of the buffering capacity of imidazole and from the equation

$$\frac{I_i}{I_o} = \frac{K_A + H^+_i}{K_A + H^+_o}$$

where I refers to imidazole concentration (11) This is less than that found by direct ΔpH measurements but considerably more than that found by the 9 A A technique

VESICLE DYE BINDING Membrane changes can also be monitored by binding of a dye such as acridine orange (AO) This has been shown to undergo binding dependent quench of fluorescence in various vesicle preparations (12) including hog gastric vesicles and frog gastric vesicles (13) There are several noteworthy features about these reactions Since acridine orange is positively charged binding is to negative sites in duced by the addition of ATP The signal reproduces many of the proper

ties already established by the pH electrode i e K^+ dependent en hanced by internal K^+ dissipated by nigericin and other ionophores (Fig 8) It depends on intact vesicles but is not quantitatively related to the amount of H^+ uptake Thus although hog vesicles take up about 20 times more H^+ than frog the AO signal is approximately the same Another feature of the AO response is that it is partially osmotically sensitive indicating that not all the response is due to binding

AO thus senses the development of negative binding sites in the vesicle membrane that are related but not quantitatively to H^+ (or OH^-) movement across the vesicle membrane The site being sensitive to buffer is in the outside region of the membranes

ACTION OF SCN SCN action has been a constant problem in gastric phy siology for more than 30 years The use of gastric vesicles perhaps might be expected to solve the mechanism of action of this compound When vesicles are placed in a KCl medium and ATP is added based on the K^+:H^+ antiport (or K^+ OH symport) hypothesis prior to proton gradient develop-

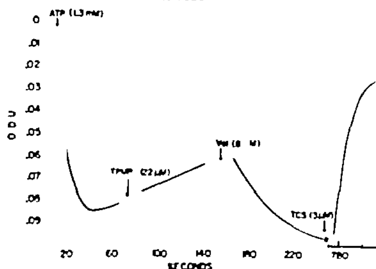


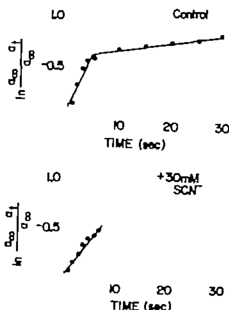
Fig 8 The effect of ATP on acridine orange absor bance showing that whereas $TPMP^+$ has little effect valin omycin increases the response which is sub- sequently dissipated by TCS

ment KCl has to enter the vesicle A considerable amount of evidence has accumulated that in the absence of ATP vesicle conductance to either K^+ or Cl is low This suggests that a KCl symport pathway is involved in K^+ uptake for the transport reaction

Accordingly inhibition of transport by the vesicles could occur (a) by inhibition of the ATPase (b) by increasing the H^+ leak and (c) by inhibition of the KCl symport When vesicles are placed in KCl solution for a short time and then ATP is added H^+ uptake occurs in two phases as shown in Fig 9 with two clearly different rate constants

As shown in Fig 9 SCN does not affect the fast uptake but blocks the slow uptake phase SCN^- added subsequent to the development of an H^+ gradient does not increase the leak of H^+ nor does SCN inhibit the ATPase of hog gastric vesicles Thus SCN interferes with the KCl symport step in some manner The AO signal is also sensitive to SCN being completely abolished if 30 mM SCN is added prior to the addition of ATP

Fig 9 An exponential analysis of H^+ uptake by non equilibrated vesicles showing two kinetic constants The slower of these is blocked by SCN



If the SCN is added subsequent to the signal the decay of the signal is somewhat faster than usual but not as fast as is obtained with ionophores This indicates that SCN^- inhibits a forward step in the transport process but does not induce sufficient leak to account for the inhibition

SCN has been shown to inhibit mitochondrial F_1 ATPase at concentrations which also inhibit gastric secretion There is also considerable dispute as to whether all the HCO_3 activated SCN inhibitable ATPase present in gastric mucosa is localized to mitochondrial fractions or is also present in the plasma membrane

The action of SCN in inhibiting the AO response reducing the KCl entry dependent component of H^+ uptake and the sensitivity of the AO response to external buffer indicates that the simple H^+ K^+ antiport model might require elaboration

In the intact mucosa another component might be required for activating an OH /Cl antiport A model consisting of an OH /Cl K^+/H^+ antiports in series is difficult to distinguish experimentally from a model consisting of a K^+/OH symport and H^+/Cl symport in series In these models an intermediate compartment is produced A condensed version of these is also shown in Fig 10 where the OH /Cl exchange component is visualized as a binding site reaction If AO binds to the OH and SCN displaces OH then the Cl available for KCl symport would be reduced and the AO signal would be inhibited (Fig 10)

EFFECT OF Cl REMOVAL The action of Cl removal and SO_4 or isethionate substitution could perhaps be understood by restriction of K_2SO_4 entry as compared to KCl entry Tracer uptake measurements show that this is the case (5) However even after 72 hrs incubation H^+ uptake is still slower in the absence of Cl and remains osmotically sensitive Fig 11

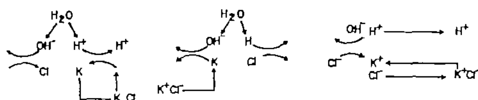


Fig 10 Possible models involving intransmembranal compartments of H^+ OH^- or K^+ classified as series models or a model involving an OH^-/Cl exchange site on the membrane surface

compares control conditions where KCl is present on both sides of the membrane to the situation where SO_4 is internal and Cl external showing a reduction in rate and magnitude of H^+ uptake Also in this figure the presence of SO_4 only on both sides of the membrane shows a slower uptake of H^+ The addition of Cl in the presence of ATP increases uptake The uptake is difficult to dissipate with protonophores valinomycin or nigericin but is dissipated by tributyltin (a Cl /OH^- antiport) In the series type of model SO_4 would not substitute for Cl with consequent accumulation of OH or H^+ in a membrane compartment In the single model SO_4 would reduce the cycling of K^+ It is surprising that an H^+ gradient is found in the vesicle preparation since intact piglet mucosa does not secrete H^+ in the presence of SO_4 (14) Certainly the permeability of the membrane vesicles to K_2SO_4 is very low

CELL MODEL It is possible to provide a rather simple conceptual scheme for transport in hog vesicles and piglet gastric mucosa The vesicles are visualized as containing an active K^+H^+ antiport and a passive KCl symport In the intact parietal cell the basal surface contains the well known $Na^+ + K^+$ ATPase with 3 2 Na^+ K^+ coupling An neutral NaCl symport on the same cell surface allows the cell to accumulate KCl The excess Cl moves

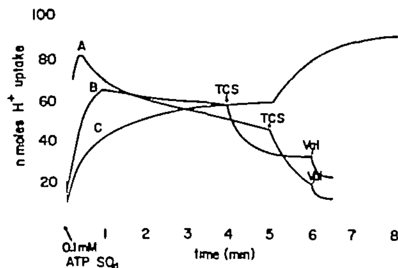


Fig 11 The H^+ uptake of hog vesicles in the KCl equilibrated condition (A) K_2SO_4 inside KCl outside (B) and with K_2SO_4 on both sides of the membrane (C)

across the luminal membrane by a Cl uniport the circuit being completed by Na^+ moving across the paracellular shunt This predicts that active Cl transport is ouabain sensitive and Na^+ dependent the former being true of piglet (14) the latter of frog (15)

The accumulated KCl is used for the K^+ H^+ exchange by having the KCl symport present on the luminal surface allowing KCl exit followed by K^+:H^+ exchange (Fig 12)

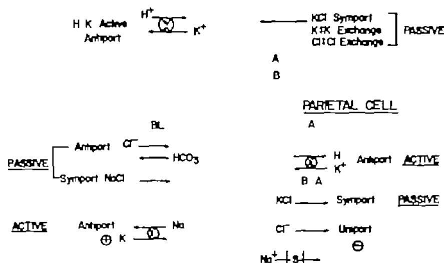


Fig 12 A general model for gastric vesicles and the gastric parietal cell using the H^+ K^+ and Na^+ K^+ ATPases to drive all the known transport processes

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Properties of a K^+/H^+ Exchange ATPase from Dog Gastric Mucosa

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ABSTRACT The electrically neutral ATP hydroly i driven transport of H^+ ions into vesicles prepared from dog gastric mucosa is dependant on K^+ ions in the internal phase of the vesicles The transport of H^+ ions by thi system i collapsed by nigericin indicating that the ATPase catalyse th exchange of an equivalent numbe of K^+ ions out of the vesicles for H^+ into the vesicles The effect of replacing Cl by SO_4^{2} or glucuron-at on the ATP hydrolysis driven exchang of H^+ and K^+ ions suggests that Cl is not equired fo thi process *per se* but Cl ions may be important for the transport of K^+ ion into the vesicles

INTRODUCTION It was demonstrated (1) that an ATPase system with a Km for ATP of 20 µM can be solated on ion impermeabl vesicles The ATPase activity is increased by K ions and by K^+ carrying ionophores in the presenc f K^+ ions as reported for imilar ATPase system f om bullfrog gastric mucosa (2) On addition of ATP the ATPase system from dog gast ric mucosa (1) catalysed an alkalinisation of the outer medium which was accompanied by an equivalent acidification of the internal vesicl phase Thus the pH chang observed in the outer medium (pH_o) was not the esult of the formation of basi substance but was formally equivalent to the ATP hydrolysis driven transport of H ion into the vesicles It was noted that important difference existed between the ATP hydrolysi driven entry of H^+ ions into vesicles f the dog gastric microsomal fr ction and the ATP hydrolysis driven proton translocation observed in sonic parti les derived from mitochondria (3 4 5) In order to observe the translocation of H ions across the membrane of submitochondrial particles it is necessary to provide a mechanism for the movement of a counter ion which collapses the electrical component of the proton motive potential and thi may be accomplished by the use of valinomycin in a medium containing K

ions It seemed probabl ther for that the ATPase system from dog gastric mucosa catalysed an electrically neutral transport of H^+ ions accompanied by the transport of a counter ion. The finding that neither valinomycin in a medium containing K^+ ions nor FCCP when added alone collapsed the pH_o changes supported this view and suggested that the membrane vesicles were impermeabl to the counter ion Furthermore it ruled out the possibility that the counter ion i a mobile charged substanc present in th membrane as proposed to explain the backlash phenomenon in mitochondria (3) Similar conclusions have been reached for an ATPase system from hog gastri mucosa (6)

The observation that K^+ ions stimul te the ATPase (1 2) and that addition of ionophore result in a further timulation suggested that K^+ entry into the vesicles is important in maintaining optimum activity and that K^+ ions are the counter ion for H^+ ion transport Thi paper d scribes experiments which support this view

METHODS Oligomycin wa purchased from Sigma valinomycin from Calbiochem, FCCP and gramicidin D from Boehringer and nigericin was a gift from Eli Lilly

Dog gastric mucosa was obtained from control animal (Beagles 10-22 Kg) used in toxicological studies The antral and cardiac regions wer cut away and the glandular mucosa wa removed from the muscle by scraping It was hopped finely in a medium containing 250 mM sucrose buffered at pH 7 4 with 10 mM Tris HCl (sucrose Tris HCl) to give a suspension containing 20-25 g wet weight of tissue per 100 ml When K^+ ion containing vesicles were prepared sucrose was replaced by 150 mM KCl The tissue wa homogenised for 5 seconds in a Silverson tissue homogeniser followed by 5 strokes at 1500 2000 rpm in loos fitting t flon glass homogeniser Differential centrifugation was carried out to give esid es at 500 g x 10 minutes 20 000 g x 20 minutes and 150 000 g x 90 minutes The 150 000 g esidue (microsomal fraction) was washed in sucrose (250 mM) medium and resuspended in the same medium except when homogenisation wa done in a KCl medium when two washe wer employed All manipulations were done at 0-4°

The reaction cell (volume 4 ml) and electrode system used in measurements of pH was as desc ibed by Mitchell and Moyle (4) ATP hydrolysis driven changes of pH_o wer measured by the ATP pulse techniqu described

by Mitchell and Moyl (5) Microsomal fraction was added to the chamber containing 150 mM KCl 5 mM glycyl glycine and 2 mM $MgCl_2$ under aerobic conditions and incubated for 15 minutes prior to the addition of MgATP in the presence of 2 5 µg/ml oligomycin Measurements wer made in the pH_o range 6 1 6 15 at 25° In this pH rang no net acid i produced when ATP i hydrolysed to ADP and Pi (7) ATP was added a 10 µl of a solution containing 10 mM Na_2ATP and 10 mM $MgCl_2$ referred to as the MgATP solution Valinomycin FCCP nigericin and oligomycin wer added as solutions in methanol (1-10 µl) Protein concentration was measured by the method of Lowry *et al* (8)

RESULTS Figure 1 illustrates a typical experiment in which we have examined the effect of nigericin on the time-course of ΔpH_o following th addition of MgATP (100 nmoles) to a suspension of microsomal vesicles in the pH rang 6 1 - 6 15 Trace A records the pH_o change in the KCl medium in the absence of nigericin Following treatment of the vesi les with nigericin (2 5 µg/ml) the extent of the pH_o chang observed during the hydrolysis of MgATP i reduced and decay rapidly to the base line Nigericin catalyses a tightly coupled electrically neutral exchange of H^+ and K^+ ions in membranes from various sources (9) Thus the collapse of ΔpH by nigericin following addition of MgATP indicates that the ATPase system catalyses the ATP hydrolysis driven transport of H^+ ions into the vesicles and an equivalent transport of K^+ ion out of the vesicl

If K^+ ions are the counter ion for H^+ ions the ATP hydrolysis driven pH_o hanges should be decreased in the absence of K^+ ions When the KCl

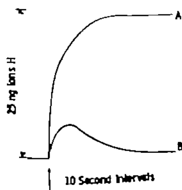


Figure 1 shows the time-course of ATP hydrolysis driven ΔpH_o in a suspension of microsomal vesicles in the KCl medium described in Methods (Trace A) Trace B shows the time-course of ΔpH_o in the presence of nigericin (2 5 ng/ml) MgATP (100 nmoles) was added indicated by rrows
Protein concentration 0 7 mg/ml

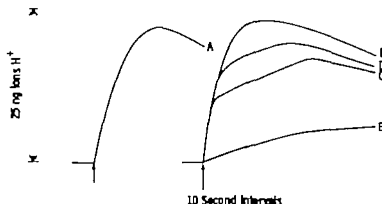
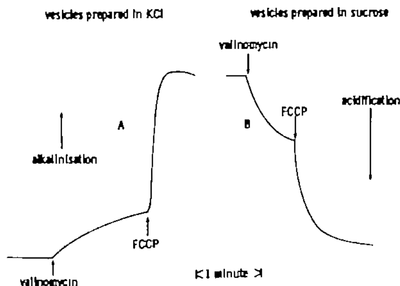


Figure 2 shows the time-course of ATP hydrolysis driven ΔpH_o in suspensions of microsomal vesicles in media of varying K^+ concentration Trac (A) 150 mM KCl (B) 150 mM choline chlorid (C) 10 mM KCl + 140 mM choline chloride (D) 20 mM KCl + 130 mM cholin chlorid (E) 50 mM KCl + 100 mM choline chloride MgATP (100 nmoles) was added indicated by arrows Protein concentr tion 0 78 mg/ml

medium i substituted by NaCl o cholin chloride medium both the extent and rate of the chang of pH_o is greatly educed. In Figure 2 the pH_o changes observed in KCl medium (Trace A) and choline hloride (Trace B) are compared Traces C D and E how the effect of increasing K^+ concentr tion on pH_o changes during ATP hydrolysi in subsequent experiments with the same preparation In the presence of 10 mM and 20 mM KCl a rapid phase and a slow phase of the time-course of ΔpH_o is observed The rapid phase probably epresents the transport into the vesi les of H^+ ions in exchange for equilibrated K^+ ions We interpret the slow phase as being limited by the re-entry of K^+ into the vesicles

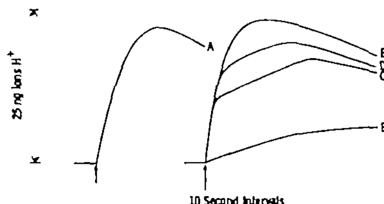
If the ve icular ATPase system i prepared with 150 mM KCl repl cing sucrose microsomal vesicles can be obtained which retain KCl. Thi is demonstrated in Figure 3 When vesi les prepared in KCl and washed in sucrose ar suspended in a choline chloride medium (Trace A) addition of valinomycin results in an alkalinisation of the medium and a more rapid alkalinisation i observed on adding FCCP Thu the exit of K^+ from the vesi les is limited by the movement of a counter ion but when the vesicl membranes ar made freely permeabl to H^+ ion these can enter rapidly in exchange for K ions The converse i observed when vesicles prepared in sucrose (low K ion content) ar placed in KCl medium (Trace B)



Figur 3 shows the time-course f ΔpH_o when valinomycin (1 25 mg/ml) and FCCP (4 5 x 10^{-6}M) were added as indicated by the arrows to su pensions of microsomal vesicles (A) V les prepared i KCl medium and suspended in 150 mM choline chlorid ; protein concentration 0 7 mg/ml (B) Vesicles prepared in sucrose medium and suspended in 150 mM KCl; protein concentration 0 55 mg/ml

Figure 4 (Trace A) shows that when vesi les with a high K^+ content are suspended in a K^+ free medium the addition of MgATP esults in a rapid chang f ΔpH_o which contrast with the lugg h changes observed when low K^+ containing vesicles are suspended thi medium (Figur 2) Trace B shows the luggish hang f ΔpH in sponse to a further addition of 100 nmoles MgATP Thi suggests that the K ions retained at high concentr tion in the vesicles have been pumped into th outer phase during the hydrolysi of the fir t addition of MgATP and insuffi ent K is tained within the esi l to support the K^+/H exchang with a second addition of MgATP

The mechanism f gastric ac d secretion involve the transport f Cl ions in addition to H^+ ion by the lumenal membrane f the parietal cell Sinc the tr nsport of th ions losely linked we have examined the ffect of Cl ion on the ATP hydroly i driven chang f ΔpH_o Replacement of Cl i the suspending medium by SO_4^{2} or glucuronate does not abolish the observed ΔpH hange in esponse to MgATP However illu tr ted Figur 5 the hange in ΔpH_o in K^+ glucuronat medium ha ha acteristic low phase when compared with hanges in KCl medium



Figur 2 shows th time-course of ATP hydrolysis driven ΔpH_o in suspensions of microsomal vesicles in media of varying K^+ concentration Trac (A) 150 mM KCl (B) 150 mM choline chloride (C) 10 mM KCl + 140 mM choline chloride (D) 20 mM KCl + 130 mM choline chlorid (E) 50 mM KCl + 100 mM choline chloride MgATP (100 nmoles) was added as indicated by arrows Protein concentration 0 78 mg/ml

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thi cannot be achieved by valinomycin or FCCP alone but only in combination it seems reasonable to conclude that thi ATPase catalyses an exchang of 1 H^+ ion for 1 K^+ ion when examined under the conditions described in this paper In a imilar system from pig gastric mucosa in which vesicles were loaded with Rb^+ it has been demonstrated that during ATP hydrolyis the exit of Rb^+ and entry of H^+ has a ratio near unity (10)

In keeping with this hypothesis it has been demonstrated that optimum proton transport driven by ATP proceeds only when K^+ ions are present within the vesicles The proton transport i greatly diminished in media in which the K^+ ions are replaced by Na^+ ions or choline ions as has been reported for the system from pig gastric mucosa (6) However when K^+ loaded vesicles are prepared proton transport proceeds rapidly irrespective of the cation in the external medium

If the K^+/H^+ ion exchang ATPase located on the membrane vesicles from dog gastric mucosa i the system responsible for the transport of H^+ ions by the lumenal membrane of the pari tal cell it seems reasonable to suggest that the mechanism for the transport of Cl ions might be associated with these vesicl membranes The finding that th ATP hydrolysi driven transport of H^+ ions is only moderately reduced when Cl^- ions are replaced by glucuronate or SO_4^{2} ions suggests that Cl^- ions ar not important in the exchang of H^+ and K^+ ions per se However the slow phase observed in the time-course of ΔpH_o in th absence of Cl is similar to that observed in low K^+ media (see F g 3) This suggests that the equilibration of K^+ during the 15 minutes preincubation prior to the addition f MgATP is decreased in the absenc of Cl ions Thi might be explained if the natural permeability of the vesicles to Cl i greater than glucuronate or SO_4^{2} However it is possibl that K^+ entry into the vesicles via a transport pathway i dependant on the presence of Cl ions The cotransport of K^+ and Cl ions across the lumenal membrane of the pari tal ell in parallel with th H^+/K^+ exchange ATPas might be an important mechanism for the secretion of HCl

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MEASUREMENT of BIOGENIC AMINES

using

cation exchange chromatography and fluorimetric assay

By

COLIN ATACK

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This review relates in particular to the following papers which will be referred to by their Roman numerals:

I Atack C and Magnusson T A procedure for the isol tion of noradrenaline (together with adrenaline) dopamine 5-hydroxytryptamine and hi tamine from the same tissue sampl using a single column of trongly idi cation ex hange resin.
Acta pharmacol et toxicol I press (1977)

II Atack C and Lindqvist M.: Conjoint nati e and orthophthaldialdehyde--condensate assays for the fluorimetri determination of 5-hydroxyindoles I brain
Naunyn-Schmiedeberg s Arch Pharmacol 279 267 284 (1973)

III Atack C V The determination of dopamine by a modification of the dihydroxyindol fluorimetri assay
Br J Pharmac. 48 699-714 (1973)

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CONTENTS

ABBREVIATIONS

Some commonly used abbreviations are the following

CNS	central nervous system
CA	catecholamines
NA	noradrenaline
A	adrenaline
DA	dopamine
5-HT	5-hydroxytryptamine
Hm	histamine
NM	normetanephrine
M	metanephrine
3-MT	3-methoxytyramine
Spd	spermidine
DOPA	dihydroxyphenylacetic acid
5-HTP	5-hydroxytryptophan
Hd	histidine
DOPAC	dihydroxyphenylacetic acid
DOMA	dihydroxymandelic acid
HVA	homovanilli acid
5-HIAA	5-hydroxyindole-3-acetic acid
HI	hydroxyindol
DHI	dihydroxyindole
THI	trihydroxyindole

Terms used i fluorimetric assay procedures (such terms i inverted commas indicate actual fluorimetric recordings)

St	tandard
RB	reagent blank
RB-rev	reagent blank reverse procedure
PS	purification sample from a blank eluate
PISt	purification internal standard from a blank eluate
PB	purification blank from blank eluate
PB-rev	purification blank reverse procedure
S	sample
ISt	internal standard
TB	tissue blank
TB-rev	tissue blank reverse procedure
TBf	faded blank procedure
TBu	unoxidized blank procedure

OPT	orthophthaldialdehyde
UV-light	ltra-viol t light
EDA	ethylenediamine
EDTA	disodium thylene diamine tetraacetate

SUMMARY

Consideration i gi en to some of the many methods used for the purification and subsequent fluorimetri determination of the biogeni amines: noradrenali and adrenaline dopami 5-hydroxytryptamine and hi tamine Particular emphasi is given to the methods utili ed i the laboratories i the Department of Pharmacology Uni ersity of Gothenburg

Purification of the amines i accomplished on short columns of strongly acidi cation exchange esi F om one tissue sampl the extracted amines are isol ted on singl column i to separate luates of small olume: noradrenaline plus adrenaline i 7 ml dopamine i 3.5 ml 5-hydroxytryptamine i 7 ml and histamine i 4 5 ml with easonably high recoveries of 75 100% depending on the amine Excellent separation i achieved from non-basi compounds. Th procedure i compl ted i a working day and several of the amine metabolites including tyrosine DOPA 3-methoxytyramine tryptophan 5-hydroxytryptophan 5-hydroxyindole-3-aceti acid and hi tidine may also be obtained i luates suitabl for assay

The fluorimetric procedure used for assaying 5-hydroxytryptamine iso 5-hydroxytryptophan and 5-hydroxyindole-3-aceti acid is conjoi t nativ and orthophthaldialdehyd (OPT)-condensate assay with tissue blank procedure common to the component assays The conjoi t assay has combined th advantages of the simpli ity and rapidity of the nati assay which is adequate for so many purposes with the high sensitivity of the OPT-condensate assay; and also provides partial double-check on the accuracy and specifi ity of the indol determination.

For hi tamine the OPT condensate assay is used and the analytical procedur i especi lly valuabl for studies of hi tamine i brai tissue because of its complete separation from spermidine i the column procedur

The noradrenaline and adrenaline are assayed by trihydroxyindol (THI) assays: using either ferricyanid as oxidant plus ascorbi acid as reducing/ / tabilizing agent; or especially when highest sensitivity is required ferricyanide as oxidant plus sulphite and BAL as reducing and tabili ing agents

Dopamine i measured by our new more sensiti ersion of the dihydroxyindol (DHI) assay procedur which has the added advantage of greater simpli ity and reliability ontributed by the self-regul ting nature of the oxidation of dopamine i alkaline sol tion by ferricyanide

The combination of purification and assay procedures makes possibl the measurement of ery small amounts i the region of 10 ng of each amine per tissue sampl

INTRODUCTION

The biogeni amines noradrenaline adrenaline dopamine 5-hydroxytryptamine and hi tamine are very widely distributed in animal tissues (for CAs see Holzbauer & Sharman 1972; for 5-HT see Garatti I & Val elli 1965; Erspamer 1966; for Hm see Vugman & Rocha SII 1966) Numerous studies have been concerned with the ralson d être of the amines I different tissues (see g Euler 1956; Garattini & Valzelli 1965; contributions to the 1st 2nd and 3rd Symposi on Catecholamines edited by Krayer 1959 Acheson 1966 and Usdi & Snyder 1973; and contributions I the Handbook of Experiment- l Pharmacology" sub-edited for 5-HT by Erspamer 1966 for Hm by Rocha Silva 1966 and for CAs by Blaschko & Muscholl 1972) and an increasing proportion have focussed on thei rol as putative neuro-transmitters I the mammalian central nervous system (see e.g bov references; also Hornykiewicz 1966; Carlsson 1967; Bloom & Giarman 1968; Andén Carlsson & Häggendal 1969; Green 1970; Snyder & Taylor 1972; Costa Gessa & Sandler (Eds.) 1974; and Axelrod 1974) These tudies hav frequently necessitated accurate quantitation of the amines by analytical procedures which are adequately sensitiv and specific

Analytical techniques gener lly invol quantitative extraction purification and concentration followed by quantitati assay Fluorimetri analysi of the biogeni amines I the technique most widely used t present and this eview i concerned with the procedures used for purifying and concentrating these amines and thei subsequent fluorimetri assay

Analytical Methods for Biogeni Amines P ior to the mid 1950 bioassays wer most commonly used and provided much of the early information on the biogeni amines (for CAs see Gaddum 1959 Collinghan & Cass 1963 Van 1966; for 5-HT see Garattini & Valzelli 1965 Erspamer 1966; for Hm see Code & McIntire 1956 Vugman & Rocha Silva 1966). With thei sensitivity and specifi ity could be realised the presence and importance I the CNS of NA (see Vogt 1954) 5-HT (see Twarog & Pag 1953; Ami Crawford & Gaddum 1954) and Hm (see Harris Jacobsohn & Kahlson 1952; Adam 1961) However the use of such biological measuring devices required considerabl expertise and they were more time-consuming and difficult to tandardi compared with physical methods of assay Neverthel ess because of the superior sensiti ity of bioassays often of the order of 10^{-9} to 10^{-10} g these more specifi assays were usually preferred to

the physical spectrophotometric (colourimetri) assays with sensitivity generally of th order of 10^{-6} g (see Persky 1955; Hanson 1966; McInti 1966)

In the 1950 s the use of fluorimetric assays combined the advantages of physical methods with a sensitivity similar to that of bioassay and thus to large extent supplanted lourimetry In analytical procedures using these types of assays similar methods for extraction and prior purification were often used to improve overall sensiti ity and specificity

With the introduction i 1955 of the spectrophotofluori meter designed by Bowman (Bowman Caulfield & Udenfriend 195?) fluorimetri assays became easier and thei specificity could be used to the full (see Udenfriend 1962 1969) I particular they enabled the importance of DA in the CNS to be reali ed (see Carlsson 1959b) A tremendous surge in resear h relating to the biogeni amines in the CNS ensued with the concomitant development and increasing use of agents for manipul ting the biogeni amines (see e g Pl tscher 1973) Many of these agents are tructurally simila to the amines and may cause inc easing problems f specifi ity i the bioassays; whereas fluorimetry is a physico-chemical technique with soci ted greater flexibility g wavelength combinations and the bility to record spectra providing greater disc imination and a high degree of specifi ity has been mai tained Thi i turn has led to great dependence on fluorimetry over the last 20 years

During thi period ther analytical techniques hav been i troduced Radiochemical analyses have been dev loped for the biogeni amines i which the inherent specifi ity of isotopic l belling is combi ed with the high sensitivity of assay by measuring radioactivity with a liquid sci tillation spectrometer; and thus f i ly simple extrac tion and purification procedures can often be used (se Beaven 1975) A radioimmunoassay has been described for 5-HT (Peskar & Spector 1973; and lso for normetanephrine by Peskar Peskar & Levine 1972) but i general there are difficulties in dev loping antibodies to the biogenic amines

Enzymati isotope dil tion derivati e assays hav been much more widely used for the amines: the enzyme preparations are mor specifi for the assays of 5-HT nd Hm than for the CAs nd consequently more purification i required for the CA analyses to increase overall specifi ity These analyses have been used for the CAs i g. blood plasma (see Engelman 1972; Passon & Peuler 1973) and br i (Coyl & Henry 1973; Cuello Hiley & Iversen 1973); and for 5-HT i brain (see Saavedra Brownstein & Axelrod 1973) They hav proved particularly valuabl for measuring Hm i nervous tissue especi lly befor the problems of inadequat specificity i the fluori metri analyses were overcome and emai the most sensiti type of assay av il bl for Hm of the order of 10^{-10} to 10^{-12} g (see Snyder Baldessarini & Axelrod 1966; Kobayachi & Maudsley 1972; Snyder & Taylor 1972) A cautionary note has been expressed about the versatility of such radiochemical techniques to neuropharma ologi ts concerning the isk of enzyme interaction with the drugs or their

metabolites used for manipul tion f amine homeostasi (Costa Koslow & LeFevre 1975). The use of chemicals instead of enzymes to catalyze the transfer of the radio-isotope i till largely undeveloped for assaying biogeni amines (see Beaven 1975)

Analysis of the biogeni amines using high pressur liquid chromatography sometimes preceded by ion-pai extraction (Modi & Johansson 1971; Person & Karger 1974) i rec i ing increasing ttention as means of purifying and isolating biogeni amines It has been coupled with g the UV-detector or the more sensitiv electro-chemical detector for assaying the amines with overall sensitivity of the order of 10^{-9} to 10^{-11} g for measuring CAs (g Refshauge Kissinger Dreiling Blank F eeman & Adams 1974) and 5-HT (g Chilcote 1974; see lso McMurtrey Meyerson Cashaw & Davis 1976)

The most rapidly developing of the analytical techniques availabl for tudying the monoamines and thei m tabolites ar without doubt those based on purification and isol tion using gas-liquid chromatography (GLC) whi h contributes ery high specificity This has been di ectly coupled with suitabl detecting devices for assaying the amines including flame ionization detection (FID) for the biogeni amines (g Fales & Pisano 1962; Maruyama & Takemori 1971 1972); electron capture detection (ECD) for the monoamines (e g Karoum Cattabeni Costa Ruthven & Sandler 1972; Lhuguenot & Maume 1974); and mass spectrometry (MS) Mass spectrom try i i itself ther insensitiv but has been greatly improved by the dev lopment of the alternating voltage ccelerator (AVA) with a singl ion detector (SID) or multipl ion d tector (MID) and the esulting assay technique which has been termed "mass fragmentography gives greatly increased sensiti ity and finger pri t" identification (Sweeley Elliott Fries & Ryhage 1966; Hammar Holmstedt & Ryhage 1968; see Hammar & Hessling 1971; Gordon & Frigeri 1972) Since it is generally directly coupled to GLC column which has great power of separation th term mass fragmentography has also been used to define the complete analytical procedure It increasingly meets the need to measure amines i smaller amounts of tissues g discrete brai areas where total amounts of amine ar too low for quantitation using fluorimetri analysi (see Costa i l 1975)

The sensitivity of mass fragmentography is of the order of 10^{-11} to 10^{-12} g for CA (Koslow Cattabeni & Costa 1972) and 5 10^{-11} g for 5-HT (Cattabeni Koslow & Costa 1972). The i tegration of GLC with MS llows rapid through-put" of samples e.g up to 50 samples per day with 4 amine determinations per sampl (Costa et l 1975) However mass fragmentography i rather expensive and ulnerabl not only because of the complex mechanisms used but also because large number of steps are involved: tissue extraction; preliminary conventional purification and concentration g by sol ent partitioning unless ery small amounts of tissue are extracted derivati tion procedure to form more vol til derivati es of the amines; and concentration of the derivative i to a small volume (a few µl) before i jection into the GLC; and consequently ecoveries tend to be

low Thus i ternal standards must be used throughout the procedure for each sampl and a correction made to obtai more accurate quantitative values Fluorimetri analysis requires less steps extrac tion usually being followed by a single purification and concentration procedure prior to assay and ar probably to be regarded as procedures more suited for routine use i the majority of laboratories

Fluorimetri Analyses of Biogenic Amines: Fluorimetric analysi will frequently provide the sensiti ity and specificity equi ed for the tudy of the biogenic amines. However there may follow the task of selecting th type of assay and prior purification procedur to use before further choosing from the formidabl number of modifications which ar available Some f the factors influencing the choice are the existing expertise the availabl equipment technical assistance and i particular the overall sensiti ity and specificity required Many published analytical methods present a combined purification and assay procedur which i particularly suited to the author s interest For different tissues or circumstances another combination e g a different purification procedure used with the same type of assay may be preferabl Thus in this eview the fluorimetri assay procedures and many of thei modifications are presented i separate sections f om a more general consideration of prior purification procedures Nevertheless when actually using complete analytical procedur the assay should be optimally adjusted to the eluate obtained from the purification procedur Examples ar gi en of the procedures used i our own laboratories in the Department of Pharmacology University of Gothenburg

METHODOLOGY

This review el tes to procedures used i fluorim tri analysis for which an e ellent account of principles and practical considerations may be found i the monographs entitled Fluorescence Assay in Biology and Medicine olumes I and II by S Udenfriend (1962 1969) In thi review particular attention is gi en to some of the methods recently developed i the Department of Pharmacology Uni ersity of Gothenburg and for details of materials and methodology used the reader i eferred to the original papers (I II and III)

REVIEW and PRESENT PROCEDURES

n A PURIFICATION of BIOGENIC AMINES prior to FLUORIMETRIC ASSAY

Introduction: The types of procedure most ommonly used for purifying and oncentrating biogeni amines prior to fluorimetri assay tilize hromatography: on trongly acidi (sulphoni) cation exchange resins (g. of the Dowex 50 type) or weakly acidi (carboxyli) cation exchange resins (e.g CG-50 type) or on alumina or by sol ent partitioning The methods were first described for purifying only a singl ami but many modifications hav subsequently been described some of which also permit the simultaneous determination of metabolites. For isolating several biogeni amines imultaneously these procedures ar often extended or combined (se below)

1 Procedures for the isolation of singl amines

1 Catecholamines For purifying NA A and DA procedures using Dowex 50 or alumina are probably most commonly used (for reviews see Udenfriend 1962 1969; Sharman 1971; Weil Malherbe 1971; Anton & Sayre 1972) Both can be simpl sensitive and eproducibl and provide very imilar results (e.g Gunn 1963) A Dowex 50 column procedure i used for reproducibly separating NA (plus A) from DA and for el ting these CA compl tely separately from non-basi compounds (see Section A 4); in preference to CG-50 type columns (Bergström & Hansson 1951; Kirshner & Goodall 1957) On alumina the CA are not separated from each other nor usually from DOPA but they can be separated from catechol acids. Alumina i generally used for recovering the CA compl tely separate from the corresponding 3-O-methyl amines (Shaw 1938; Lund 1949; Anton & Sayr 1962 1964; Drell 1970; W il Malherbe 1971) i preference to ion-exchange columns (Häggendal 1962 1962b 1963b; Mattok & Wilson 1965; Wright 1958) Recently CAs have been purified on columns of boric acid gel (Higa Suzki Hayashi Tsuge & Yamamur 1977) For most of our biological experiments mor important gains in specifi ity are obtained by recovering

separately NA plus A DA and DOPA plus tyrosine I the Dowex 50 procedure together with an lmost compl te separation of the CAs from thei 3-O-methyl derivati es than by ecovering the CA separately from thei 3-O-methylated metabolites on alumina However no single procedur gives enti ely satisfactory specificity for the fluorimetri determination of CA, and consequently alumina has often been used in conjunction with either Dowex 50 type (Costa t al 1968; Taylor & Laverty 1969) or CG-50 type procedures (W ll Malherbe 1968; Renzini Brunori & Valori 1970) for purifying CA and also for the simultaneous determination of many of thei metabolites (see also Masuoka D ell Schott Alcaraz & James 1963; Spano & Neff 1971) Acid butanol partitioning of the lkaline-l bil CA I which e-coveries tend to be low has been used lone (Shore & Olin 1958); also I combination with alumina (Chang 1964) or CG-50 columns (Fleming Clark Fenster & Towne 1965) to improve specificity Quantitative specifi paper chromatographical procedures hav also been developed (Laverty & Sharman 1965; Crawford & Y tes 1970)

5-Hydroxytryptami e: 5-HT alone is probably best purified on CG-50 type columns (for revi w see Sandler 1968; Anton & Sayr 1972) Although most other amines or not separated from 5-HT those likely to be present produc litt l terference when assaying 5-HT The procedur I more impl and rapid and 5-HT can be more easily eluted in a small volume with high recoveries using the CG-50 type columns (Berti 1961; Davi Huff & Brown 1964 Andén & Magnusson 1967) than using Dowex 50 type columns In both procedures interfering non-basi metabolites are emoved Of these 5-HIAA has been imultaneously ecovered separately from 5-HT I imple 2-step procedur using Sephadex G-10 and CG-50 type columns (Jonsson & Lewander 1970; Ahtee Sharman & Vogt 1970) The Dowe 50 procedur is useful for the simultaneous purification of 5-HT and several of its metabolites (see Section A 4) 5-Methoxytryptamine I also separated and can be collected after 5-HT (see Paper II; cf Green Koslow & Costa 1973) Techniques employing organi sol ent partitioning e g alkaline butanol for 5-HT (Udenfriend W issbach & Clark 1955 b; Mirolli 1966; Wise 1967) hav been extended to simultaneously ecover el ted ompounds by using additional sol ents (Quay 1963; Miller & Maickel 1970; Kāno 1974) and have been used I an automated method by Korf Schutte & Venema (1973) Paper chromatographical procedures hav also been used (Ashcroft Eccleston & Crawford 1965)

Hi tamine: Hm is compl tely separated from histidine nd ther non-basi compounds on cation exchange columns. It i eluted I a small volume with high ecoveries compl tely separate from spermidine using either the Dowex 50 procedure (see Section A 4; lso Green & Erickson 1964; Lorenz t al 1970) or i termediately acidi (phosphonic) cation exchangers (Kremzner & Pf iffer 1966; Medina & Shor 1966; Michaelson & Coffman 1969 Schwa tz Lampart & Rose 1970) the l tter ex hangers being preferabl for ecovering lso the Spd for assay (Mi haelson 1967) Specifi ty with these procedures is greater than with either CG-50 type columns (Adam Hardwick & Spencer 1957; Oates Marsh &

Sjoerdsma 1962; cf Gr en & Erickson 1964) or alkaline solvent partitioning alone (Shor t al 1959; Anton & Sayre 1969; Harvey 1973) which has been automated (Siraganian 1974) The amounts of Hm present I neural tissues ar normally small compared with the monoamines and ofte the lower limits needed for the sensitiv fluori metri assay ar approached However the enzymati isotopi derivati e techniques developed for Hm are adequately specifi and being mor sensitive ar now often preferred (see Snyder & Taylor 1972)

Purification Procedures General Comments

From an examination of the above and other published methods certain generalizations may be ttempted with regards to the use of the diff rent chromatographical techniques for purifying biogeni amines prior to fluorimetri assay

Using cation exchange columns the biogeni amines ar gener lly adsorbed at an acid or neutral pH The amines can easily be compl tely separated from all acidi neutral and amphoteric compounds (including g potentially interfering precursor amino acids) by using a variety of washes The generally high recoveries of amines in small eluate volumes ar unaffected by these washes which also greatly educe the non-specifi fluorescent material derived f om tissue resulting in lower TB Thes fa tors contribute to increased specifi ity

Dowex 50 type resins ar often preferabl t the CG-50 type esins for purifying an amine I situations wher greater specificity I usefully gained by separating it from ther amines. Thi is because on Dowex 50 type columns the amines are reproducibly separated yet till in small eluate olumes even when extracted from relatively large amounts of tissues whereas on CG-50 type column separ tion of amines is mor sensitive to vari tions in the electrolyte concentration of both the sampl and eluants For similar easons Dowex 50 type columns ar preferred wher further resolution of the non-basi fraction g of amino acids I simultaneously equired. The buffer or weakly acid luates often obtained from CG-50 type columns ar sometimes mor convenient for fluorimetri assay Although trong acids are generally used to lut amines from Dowex 50 type columns fluorescent material leached from the esi causes only light interference I the assays if proper precautions ar taken (Häggendal 1962a; see also Paper III) Alumina adsorption I a ery useful procedur for purifying CA being comparatively specifi I taining only catechol and not non-catechol compounds Thei adsorption I littl influenced by the salt content of the sampl making it especially valuabl I the preliminary purification of CA from urine P ocedures can be simpl and apid with high ecoveries I small luate volumes although the best conditions both for the adsorption of the alkaline-labil CA and thei elution are not agreed upon as partly evidenced by the large number of published modifications

With sol ent partitioning of amines g using salt-saturated butanol final eluates ar also small However amines

or lost in washes which or used to improv upon the incompl te separation of amino acids and to reduce blank fluorescence in the assays Also amine recoveries can be considerably influenced by sampl constituents often necessitating subdivi ion of each tissue extract with resultant loss of sensitivity one half being used with added authentic amine as a check on recovery Thus for purifying biogeni amines prior to fluorimetric assay procedures other than solvent partitioning or often preferred (see e g Weil Malherbe 1971; also section A 4) which or simple and of short duration but in which greater specificity and sensiti ity or achieved Paper and thin-layer chromatography or with certain noted exceptions generally only used for qualitative and semi quantitativ amine analyses

Procedures for the simultaneous isol tion of several amines: For simultaneously purifying sever l amines most availabl procedures are extensions or combinations of the methods described above for single amine with the same inherent advantages and disadvantages.

Using singl olum of Dowex 50 esin as described (most the entire content of NA (plus A) DA 5-HT and Hm original ly present i the tissue sampl is purified and concentrated into separat generally small final luate volumes with high recoveries and specificity and many of the amine metabolites can also be collected

Separation of the amines has also been achieved on columns of intermediately acidi (phosphoni) cation ex hange resins (Endo & Ogura 1973) and on weakly acidic cation exchange resins (Holman Angwin & Barchas 1976) and also on alumina followed by CG-50 columns (Karasawa Furukawa Yoshida & Shimuzu 1975)

Sol ent partitioning has been used lone or as the first purification step after extractions using either acid solvent extractions for CA and 5-HT (Mead & Finger 1961; Brownlee & Spriggs 1965; Maickel Cox, Saillant & Miller 1968; plus 5-HIAA Welch & Welch 1969; Miller Cox Snodgrass & Maickel 1970); or alkaline sol ents for 5-HT and Hm (Cals 1961); or sol ents at both pHs for all these biogenic amines (Sadavongvivad 1970) Recoveries of the CA tend to be low Acid sol ent partitioning of the monoamines has been followed by either alumina and alkaline sol ent partitioning (Ansell & Beeson 1968; plus 5-HIAA Cox & Perhach 1973; plus 5-HIAA and HVA Haubrich & Denzer 1973) or CG-50 (Fleming et l 1965; Kariya & Aprison 1969) or by series of columns of weak cation and weak and trong anion exchange esins for monoamines 5-HIAA and precursor amino acids (Smith Lane Shea McBride & Aprison 1975) or Dowex 50 (plus DOPA Wiegand & Perry 1961) to improve specificity but sensiti ity is decreased being the product of losses i different methods Alkaline sol ent partitioning of the non-CAs has been preceded by purification of the monoamines on a CG-50 type column (Barchas Erdelyi & Angwin 1972) on Dowex 50 (Cox & Potkonjak 1967) or on an alumina column (Shellenberger & Gordon 1971)

In many of these procedures sensiti ity is lost by sub-di ‹di g the extract for two different purification procedures (e g.

Sadavongvivad 1970) or for checks on amine ecoveries (g Ansell & Beeson 1968 Cox & Perhach 1973) or more commonly by sub-division of the final eluate which contains several amines (e g Barchas t 1 1972; Brownlee & Spriggs 1965; Anton & Sayre 1964; Weil Malherbe 1971; Schlumpf t al 1974) Sub-di i ion of the eluate has been avoided by the sequential fluorimetri assay i the same tube of A NA and DA (Chang 1964; see also Ansell & Beeson 1968; Shellenberger & Gordon 1971) and of 5-HT and Hm (Sadavongvivad 1970) but the ttainment of adequate specifi ity must be carefully checked

The i troduction of Dowex 50 columns by Moore Stein and co-workers for imultaneously separating a large number of amino acids eventually i an automated procedure (see Spackman Stei & Moore 1958) has proved invaluable for biochemical research. Modifi cations of the Dowex 50 type procedures hav led to the development of procedures i which t least 10 amines and related compounds of great i terest to the neuro-pharmacologist can be simultaneously isol ted i a singl Dowex 50 procedure Further modifications of this procedure to increase the number of compounds which can be separated should be possibl and the high eproducibility may eventually lead to the development of an utomated procedure.

PRESENT PROCEDURE for the PURIFICATION of BIOGENIC AMINES prior t fluorimetri assay:

Introduction: A procedure for the purification and concentration of the biogeni amines NA plus A DA 5-HT and Hm i to separate small eluates using ingl column of Dowex 50 es i described i Paper I

The development of such procedure was undertaken to provid a mor convenient method for measuring the high concentrations of Hm DA and 5-HT i ruminant and rodent mast cells. At the time no ingl purification procedure for all these amines exi ted although of the separat procedures availabl for each amine organi sol ent partitioning and more especi lly the Dowex 50 type cation--exchange column methods appeared promising as candidates for combined procedure

Dowex 50 columns were i troduced by Bertler Carlsson & Rosengren (1958) for the purification and isol tion of DA and NA plus A deri ed from brai tissue Subsequently separate Dowex 50 procedures were described for Hm (Green & Erickson 1964) and for 5-HT (Cox & Potkonjak 1967) The linking of these procedures provided a method with several useful features: after placing the tissue extract on ingl Dowex 50 column simpl washing procedures could be used to remove all non-basi compounds with irtually no effect on the ecoveries of the amines; and the total content of each amine i the tissue sampl could be resolved i to individual eluates. I prototype Dowex 50 procedure DA was el ted i 12 ml N HCl Hm i 4.5 ml 2 N HCl and after washes with water to obtai a neutral effluent

5-HT was eluted with 20 ml 0 1 N NaOH (I preference to 20 ml 4N HCl)

For studying th ole of monoamines I the CNS the original Dowex 50 column procedure for the separate el tion of NA plus A I to 8 ml N HCl and DA I to 12 ml N HCl was used (Bertler t l 1958- Carlsson & Lindqvist 1962b) I parallel with a CG-50 procedur I which 5-HT was el ted in 3.5 ml N HCl (Andén & Magnusson 1967) Using these cation exchange esins all non-bases including the precursor amino acids and 5-HIAA were removed by imple washing procedures; this frequently being of importance for neuropharmacological studies However sub-di I ion of the tissue extract nd the running of two separate olumn procedures was required and thus the combined Dowex 50 procedure offered potential advantage but the 5-HT luate was too large

By incorporating ethanol i to th eluant acids the monoamines were l ted I much smaller volumes leaving Hm ompletely unaffected but separation of the monoamines was no longer complete However by using combination of aqueous and thanol I HCl eluents the amines could agai be esol ed I to separate luates whil t retai ing small eluate olumes Thus in the final procedur NA plus A wer still luted I 8 ml N-aqueous-HCl but DA and 5-HT wer luted in ery much smaller olumes using N-ethanoli (50%)-HCl DA in 3.5 ml and 5-HT I 7 ml; the Hm eluate remai ing as 4 5 ml 2N aqueous HCl

The four-fold decrease I the DA luate volume provided a valuable contribution to over ll sensiti ity especially when coupled with the more sensitive assay for DA which has been developed for this eluate (see section E 6) For 5-HT the sensiti ity emained unchanged inc although the eluate olume was doubled the complet tissue extract could be used with saving I time and convenience

A preliminary report of this single Dowe 50 column procedure for the imultaneous purification of the fiv biogeni amines was presented (Atack & Magnusson 1970) shortly fter the only other procedur for purifying these amines was described (Sadavongvivad 1970) I which a combination of acid and alkaline organi sol ent partitionings with sub-divi ion of the eluates prior to assay was used This procedure did not hav the advantages of specificity and sensitivity obtained with the Dowex 50 procedure

The Dowex 50 procedur has now been I routine use for several years During this time a number of additional procedures have been incorporated for the purification and lution of sever l metabolites of these amines from the same column including e g 5-HTP and 5-HIAA (Lindqvist 1971); tryptophan (Bédard Carlsson & Lindqvist 1972); tyrosine and DOPA (Kehr Carlsson & Lindqvi t 1972) and 3-MT (Kehr 1974).

The use of Dowex 50 columns emains the easiest means available for compl tely separ ting NA plus A from DA. This fa tor combined with the numerous other advantages of the use of such columns (see also below) has prompted us to present the procedur I considerable practical detail (Paper I)

Procedures for the isolation of a singl amine:

Catecholamines. For NA and A the Dowex 50 procedure is the method of choice i these laboratories due to the ery high specificity contributed to the analytical procedure (see Paper I and section D 6) Important separations can include NA from DA as when measuring NA i the presence of relatively large amounts of DA i tissues such as the corpus striatum and NA from α-methyl-DA after administration of α-methyl-DOPA (Carlsson & Lindqvist 1962b) The contribution to sensitivity (eluate = 8 ml) is adequate for many purposes However shorter Dowex 50 columns may be used when separation from DA is less important the NA being eluted i a small (2.3 ml) volume when extra sensitivity i equired to measure NA and A e.g i blood plasma (Häggendal 1963)

For DA alone th Dowex 50 procedure is lso the method of choice; contributing high sensitivity due to the small (3.5 ml) eluate; and high specif ity to the overall procedure (see Paper I and section E 6) DA is completely separated from 6-hydroxy-DA which may be useful e.g after administration of 6-hydroxy-DOPA or 6-hydroxy-DA; and from NA which is important when measuring small amounts of DA in the presence of relatively large amounts of NA in tissues such as spinal cord (g Magnusson 1973)

The considerabl separation of NA plus A and of DA from their respective 3-O-methyl-derivatives (80 90% separation) can be of value e.g after MAO inhibition. The compl te separation of all non-bases is important notably DOPA which i terferes in the HI assays The procedur can be extended to isolate not only the CAs but lso some of thei metabolites including DOPA and tyrosine (Kehr Carlsson & Lindqvist 1972) and 3-MT (Kehr 1974). Longer Dowex 50 columns hav been used for the complete separation of the CAs from thei 3-O-methyl-derivati es derived from single tissue extract (Häggendal 1962b) An lternative approach i the use of two short Dowex 50 columns i parallel for purifying the amines after sub-division of the tissue extract: I one liquot of extract the CAs are destroyed by oxidation and 3-O-methyl-derivatives alone are purified and i the other the CAs and 3-O-methyl-derivati es are purified together (Carlsson & Lindqvist 1962a; Häggendal 1962a; Carlsson & Waldeck 1964) Dowex 50 columns have been occasionally used for urine and more accurate results may be obtained by purifying the eluate on second Dowex 50 column before assaying the CAs (e.g. Andén & Henning 1974)

5-Hydroxytryptamine: For measuring 5-HT alone CG-50 column procedure i which 5-HT is eluted i small (3.5 ml) volume (Andén & Magnusson 1967) is generally preferred to the more time-consuming Dowex 50 procedure for 5-HT although the additional specificity obtained by the compl te (>98%) separation of 5-methoxytryptamine (see Paper I Paper II) may sometimes be important (cf Green Koslow & Costa 1973) In both cation exchange procedures the separation of ll non-bases especially of 5-HIAA present

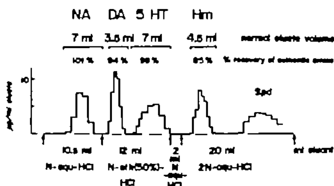
in normal brai tissue and of 5-HTP is important

However the Dowex 50 procedure becomes the method of choice for 5-HT when we require in addition the determination of some of its metabolites i the metabolic pathway tryptophan → 5-HTP → 5-HT → 5-HIAA whi h can all be recovered from the same Dowex 50 column (useful for turnover tudies of 5-HT in e g the spinal cord Carlsson Lindqvist Magnusson & Atack 1973)

A 4 2 c   Hi tamine: For measuring Hm alone the shortened form of the Dowex 50 procedure i the m thod of choi i these laboratories because it is rapid and convenient method with a small (4 5 ml) eluate and it contributes high specifi ity to the overall analysis. It is one of the few procedures in whi h ther is compl te separation of Hm from Spd which otherwise presents considerabl problems i the assay of Hm in brain (see sections C 2 3 and C 3.7); and all non-bases ar removed including Hd which ca also be collected from the same column

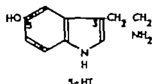
A 4 3   Procedure for the simultaneous isolation of several amines

When equiring to measur several amines from the same tissue sampl the Dowe 50 column procedure i one of the most useful methods providing analytical procedur with high sensiti ity and specifi ity The entire amount of each amine can be collected i a separate small volume with easonably high ecoveries (75 100% depending on the amine) A considerable degree of separation from interfering amines i achieved All non-bases are emoved including precursor amino acids and acid metabolites some of whi h can also be recovered for fluorimetric assay (Lindqvi t 1971 Kehr Carlsson & Lindqvist 1972) This is ery valuable for comparative studies of amines g in thyroid gland tissue (Atack Ericson & Melander 1972) Also for comparing turnover rates of the monoamines fter inhibition of the aromati amino acid decarboxylase acti ity (Carlsson Davis Kehr Lindqvist & Atack 1972) Hm can be measured i experiments designed primarily for the monoamines (see section C 3.7) Probably the most important factor is that the procedure provides the means of directly comparing several biogenic amines and a number of thei metabolites under standardized experimental conditions with increased accuracy (for review see Carlsson Kehr Lindqvist Magnusson & Atack 1972)



8 FLUORIMETRIC ASSAY of 5-HYDROXYTRYPTAMINE

Introduction: For reviews of fluorimetric assays used for 5-HT see g Udenfriend (1962 1969) Sandler (1963 1968) Garattini & Valzelli (1965) Hanson (1966) Maickel & Miller (1968) Lovenberg & Engelman (1971) and Anton & Sayre (1972) The structure of 5-hydroxy-tryptamine (5-HT)(serotonin) I 3-(2-aminoethyl)-5-hydroxyindol and its chemical properties were elucidated by Rapport and co-workers (1948 1949; see also Garattini & Valzelli 1965) They suggested simpl chemical assay could be based on its property to absorb UV -light at 293 nm Subsequently the most commonly used fluorimetric assay of 5-HT which utilises this property has been that dependent on nati fluorescenc i strong HCl; even though i the last decade some more sensitive techniques hav been evolved



NATIVE FLUORESCENCE of 5-HT I WEAK ACID

5-HT in weak acid ———→ Read t 295/340 nm

5-HT i weak acid t pH 3 exhibits the strong native fluorescenc at 295/340 nm which is characteristic of ll indoles (Bowman Caulfield & Udenfriend 1955) The various indoles produc fluorescence at simil wavelengths and even although this is maximal t different pHs (see Udenfriend 1962) because of the large number of naturally occuring indoles the assay i insufficiently specifi except for few special purposes (e.g Udenfriend et al 1955b) Blanks tend to be high due to light scatter from the proximity of activation and fluorescence wavelengths

NATIVE FLUORESCENCE of 5-HT I STRONG ACID:

5-HT i strong (ca. 3N) HCl ————→ read t 295/545 nm

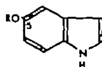
optima: The assay for 5-HT is based on its native fluorescence t 295/545 nm i trong (ca 3N) HCl (Udenfriend Bogdanski & Weissbach 1955a) and was introduced by Bogdanski Pletscher Brodie & Udenfriend (1956) for assaying 5-HT in brai tissue Increased tability of 5-HT i the strongly acid solutions has been reported when using ascorbi acid lthough its use also decreased 5-HT fluorescence

due to absorption of acti tion light and quenching of fluorescence (Andén & Magnusson 1967; Thompson Spezi & Angulo 1969a) Cysteine has lso been found to be an effective pr tecting agent for 5-HT in strong acid (Korf & V lkenburgh-Sikkema 1969) Dependent on the natur of the eluate an amount of concentr ted HCl i used to obtai maximal fluorescence Some bad batches of concentrated HCl contain impurities e g possibly F ++ions which ca destroy 5-HT (Andén & Magnusson 1967; Udenfriend 1969) Redi tilled 6N HCl ca be used as a general precaution for prepa ing weaker solutions of HCl (Andén & Magnusson 1967) Where concentrated acid is needed its suitability can be tested before use (Udenfriend 1969; see also section B 8 and paper II); and a reducing agent which protects 5-HT from the impurities e g ascorbi acid can be used in the assay (Andén & Magnusson 1967) Lowe blanks have been obtained by decreasing second order light scatter which t 590 nm is too lose to the fluorescence wavelength with polari ers crossed (Wise 1967) or singl (Adl & Hughes 1965) or with a plai glass filter (Andén & Magnusson 1967); although thei use leads to loss of fluorescence due to light absorption

B 3.2 blanks: The first TB procedur incorporated i to the assay by Boullin (1962) depended on the differential fading of 5-HT and non-specifi fluorescence i th S tube; later strong oxidants were used to accelerate the reaction (Contractor 1964) Subsequently TB procedures invol ed selecti oxidation of 5-HT i the TB tube either by ferricyanide plus UV light (Andén & Magnusson 1967) or by periodat (Korf & Sebens 1970) protection i the non-TB tubes being afforded by the reducing agents ascorbi acid and cysteine respecti ely

Additional aspects of this assay a considered below (section B 8)

B 3.3 chemistry: Indoles substituted in the 5-position with OR wher R = H or CH_3 exhibit nati e fluorescence i strong acid The fluorescenc of these 5-OR indoles activated at 295 nm is emitted at 340 nm when in weak acid; but with decreasing pH it is increasingly shifted to 540 nm whereas the fluorescence of other indoles i gradually extinguished (Udenfriend t al 1955a; Bowman t al 1955; Quay 1963) This shift in fluorescenc wavelengths has been shown to be due to excited state protonation (see Udenfriend 1969 p 8) Thus considerabl specificity i gained by assaying 5-HT i trong (ca 3N) HCl



5 OR indol

B 3 4 specificity: When using the assay for 5-HT i terfering fluorescenc of other 5-OR indoles is maximal at the same wavelengths but exhibits quantitativ differences from that of 5-HT (Quay 1963; Andén & Magnusson 1967; see also Paper II) The fluorescenc of some of these compounds i of sufficient intensity for thei individual assay g. for 5-HIAA 5-HTP and thers (Udenfriend Weissbach & Brodie 1958; Ashcroft & Sharman 1962; Quay 1963; Jonsson & Lewander 1970;

Lindqvi t 1971) Thi I an adequately specific assay for 5-HT I many tissues but I circumstances where ther 5-OR indoles occur I extracts I sufficient quantities to give measurabl fluorescence g 5-HIAA in normal brai tissue 5-HTP after admi I tration or induction ca must be taken in choosing a suitable purification procedure to separate them from 5-HT (see section A) prior to its assay The assay has been adapted to eluates obtained from most different types of purification procedure used for 5-HT g CG-50-type for br in (Andén & Magnusson 1967) and urine (Arterberry & Conley 1967) Dowex 50 for brai (Paper II) and sol ent partitioning for brain blood liver and kidney (Fi cher & Aprison 1972)

ORTHOPHTHALDIALDEHYDE CONDENSATE ASSAY for 5-HT

5-HT I very trong (ca 6.5N) HCl OPT + heat $\xrightarrow{\text{room temp}}$ read at 360/470 nm

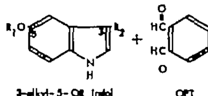
optima For assaying 5-HT Maickel & Miller (1966) I troduced the reaction with OPT I very strong (ca 6 5N) HCl during heating t 100°C for 10 minutes to produce the fluorophore; the assay being applied by Maick l Cox Saillant & Miller (1968) to measure 5-HT I brain tissue The use of cystei in the assay has resulted I consider abl increase I the reproducibility and I tensity of fluorescence (Korf & Valkenburgh-Sikkema 1969; see also Korf & Sebens 1970; Curzon & Green 1970); imil esults have been obtained using reduced gl tathione (Peuler & Passon 1973) The amount of acid requi ed to giv maximal fluorescence depends on the nature of the eluate on- entrated HCl is the acid most commonly used (g Maickel & Miller 1968; Korf & Sebens 1970- Thompson t al 1969b) although con- centr ted H_2SO_4 was subsequently preferred by Korf Schutte & Venema (1973) I appli tions of th assay various concentrations of OPT (usually dissolved I methanol) generally 0 02 0 04 mg/ml eactant sol tions and various times of incubation generally t 100°C (but c.f Section B 8) for 10 30 minutes hav been used (g Maickel & Miller 1966 1968; Miller & Maickel 1970; see also Thompson t al 1969b; Kamesu & Thompson 1971)

blanks: The first TB procedure for the assay I troduced by Korf & Sebens (1970; Korf & Valkenburgh-Sikkema 1969) i I principl imil to that used I the nati assay: invol ing oxidation of 5-HT by periodate I the TB tube with the cysteine protecting 5-HT I the non- TB tubes.

Automated ersions of the OPT-condensate assay for 5-HT (and 5-HIAA) hav been developed (Korf t al 1973; Peuler & Passon 1973)

Additional aspects of the assay are considered below (section B 8)

B 4 3 chemistry: The chemical basis of this OPT-condensate assay is that 3-alkyl 5-OR indoles where R_1 = H or CH_3 and R_2 = 2- or 3-carbon side chains can be reacted with OPT in very strong and hot HCl to form a fluorophore but the nature of the chemical reaction has not been elucidated (see Maickel & Miller 1966)



B 4 4 specifi ity: When using the assay for 5-HT i most practical applications the compounds most likely to i terfer would be the sam as i the nati e assay even lthough additional specifi ity is gained i comparison with the native assay by i tue of the substituent required in the ring-3 position However ther re quantitativ differences compared with the nati assay i the amount of fluorescence produced by the different indoles several of which give considerably mor fluorescence than 5-HT e g. mel tonin gives 3 5 times more fluor escenc than 5-HT (Maickel & Miller 1966) Fluorescence of these indoles is maximal t similar wavelengths to 5-HT (personal observation) Individual assays hav been described for 5-HIAA (Korf & V lkenburgh-Sikkema 1969; Curzon & Green 1970) 5-HTP (Tachiki & Aprison 1975) and other 3-alkyl 5-OR indoles (Miller & Maick l 1970) OPT has also been used for assaying many other compounds but under ery different conditions (see section C 2.4) For 5-HT itself the i tensity of fluorescence i substanti lly greater than i other 5-HT assays (Maick l & Miller 1966; Anton & Sayre 1972) In those circumstances where there is an expectation of other 3-alkyl 5-OR indoles i the tissue extract of which 5-HIAA and 5-HTP ar the most likely an appropri te purification procedure must be used for 5-HT prior to its assay The assay has been adapted to the eluates from most types of purification procedures e g CG-50-type for brain (Korf & Sebens 1970) and urine (Korf & Valkenburgh-Sikkema 1969); Dowex 50 for brain (Paper II) and sol ent partitioning for brai (Maickel t l 1968)

B 5 NINHYDRIN-CONDENSATE ASSAY for 5-HT

5-HT		+ heat	room temperature	read at
i buffer	ninhydri	——→		
pH 6.5		incubation time +	development time	390/495 nm
		-g 75°C for 30 mi +	60 min	

B 5.1 optima This assay for 5-HT based on its eaction with inhydri in a hot near-neutral sol tion was i troduced by Vanabl (1963) following an earlier paper-chromatographical procedure (Jepson & Stevens 1953) and was slightly modified when applied to tissues by Snyder Axelrod & Zweig (1965; also McCaman McCaman Hunt & Smith 1965) The

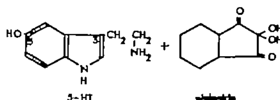
conditions used i these ersions of the assay may be considered The optimal pH of the reaction i phosphate buffer was found to be 6 5 by Vanabl (1963); other pHs used were 6.75 and 7 0 The purity of the ninhydri is important and an optimal concentr tion i the reaction mi ture of 0 8 mg/ml for pur sol tions of 5-HT was demonstrated by Vanabl (1963); for tissue eluates up to 2 mg/ml ninhydri has been used. Various combinations of incubation conditions plus development time were used: 60°C for 60 mi utes plus 30 minutes; 75°C for 30 minutes plus 60 minutes; and 100°C for 10 minutes plus 5 hours; the higher incubation temperatures giving a greater fluorescence yield (see McCaman et al 1965) However near maximal fluorescenc and increased specificity wer eported following i cubation at 60°C for 20 minutes (Quay 1968) Since fluorescence was unchanged between pH 5 and 8 it could be recorded i the same sol tion (Blau 1973)

blanks: A TB procedure i often omitted i these assays lthough Ansell & Beeson (1968) obtained a TB by adding inhydrin immedi tely before eading the fluorescence

Subsequently TB procedure was introduced by Shellenburger & Gordon (1971): after ecording the 5-HT-derived fluorescence i the S tube it was selecti ely destroyed by adding alkaline sodium sulphite reagent and the TB fluorescence was ecorded i the same tube 20 minutes l ter

Automated ersions of the inhydrin-condensate assay for 5-HT hav been developed (Blau 1973; Taylor & Crawford 1974)

chemistry: The chemi try of the rea tion between 5-HT and inhydri has not been fully established but was suggested by Vanabl (1963) to involve condensation of 5-HT with inhydri followed by cycli a-tion to form tetra-hydro-harman derivative



specificity: The eaction appears to be ery specific for 5-HT i ter ference i given by α-methyl 5-HT (ca 92% McCaman t l 1965) but most related compounds gi litt (g 6-hydroxytryptamine = 5%; buf tenine = 2 5% 5-HTP = 1 2% and 5-HIAA 0 2%) or no measur abl fluorescence (Snyder t l 1965; Quay 1968) Furthermore i terference may be decreased by using lower inc bation temperatures g 60°C for 20 minutes (Quay 1968) However problems of i ter ference i this assay hav been encountered possibly due to the presence of amino acids which are ither endogenous or hav been admi istered g p-chlorophenylalanine (PCPA)(an inhibitor of tryptophan hydroxylase)(Vanabl 1963; Ansell & Beeson 1968, Welch & Welch 1969; Anton & Sayr 1972) Also number of carboline derivatives hav nativ fluorescence at the wavelengths used i the

5-HT ninhydri assay (Quay 1968) An indi idual assay for bufotenine could possibly be developed: it produced an i tense fluorescence after a prolonged incubation of several hours with ninhydrin by which time 5-HT deri ed fluorescenc had declined to a low level (see Quay 1968). This may be useful since bufotenine is difficult to discriminate from 5-HT i the native and OPT-condensate assays (see Paper II). When analysing 5-HT purification procedures should preferably remove non-basic compounds. Published applications of the assay hav generally invol ed purification by solvent partitioning i to a final phosphate buffer luant of pH 6 5 7 0: such conditions being ideal for the di ect addition of ni hydri (g Snyder et al 1965)

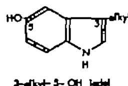
B 6 INCUBATION ASSAY for 5-HT

5-HT in K_3PO_4 buffer pH 13	+ heat e g $90^{\circ}C$ for 30 mi	cool to ⟶ room temp	read t 355/400 nm

B 6 1 optima: This incubation assay was described for 5-HT by Anton & Sayr (1972) and differs only very slightly with respect to incubation conditions from thei assay i troduced earlier for 5-HIAA (Anton & Sayre 1971). I the only published description a pH of 13 for incubation was obtained with tripotassium orthophosphate buffer the purity of which was tated to be especi lly important. For 5-HT the solution was heated at 90 C for 30 minutes and fluorescenc was found to be stable for several hours.

B 6 2 blanks: The routine TB procedure involved replacing the phosphat buffer by water i the TB tube. Similar blank values were also obtained by other means. The phosphate buffer could be added immediately before reading the fluorescence but this was not convenient for routine use. Alternati ly the 5-HT could be oxidised with periodate or hydrogen peroxide before incubation a TB procedure which the authors preferred not to adopt (Anton & Sayre 1972) but which could be useful especially if the results of thei routine TB procedure wer i doubt

B 6 3 chemi try: i this assay 3-alkyl 5-OH-indoles where the 3-alkyl side chai has erial substituents can be incubated at high pH to form fluorophores. However the chemical nature of the reaction has not been fully elucidated although Anton & Sayre (1971) suggested it may be based on an aldol -lik addition invol ing condensation of several molecules of the indol to form a fluorescent polymeric product



3-alkyl-5-OH indol

B 6 4 specificity: When using the assay for 5-HT compounds found to give considerabl interfering fluorescence were 5-HIAA 5-hydroxytryptophol

N-acetyl 5-HT (which all gav greater fluorescence than 5-HT) and 5-HTP; whereas no fluorescence was obtained from 5-methoxyindoles (Anton & Sayre 1972) (bufotenine could also be expected to i terfere; and the effect of 6-hydroxyindoles was not given) In the ersion of the assay optimized for 5-HIAA fluorescence i tensity was reported to be considerably greater than is obtained i the corresponding nativ and OPT assays (Anton & Sayre 1971) In those ircumstan es where ther 3-alkyl 5-OH-indoles ar expected to be present i the extract 5-HIAA and 5-HTP agai being the most likely an appropri te purification procedur can be used for 5-HT prior to its assay This assay seems simpl and straightforward especially for applying to 5-HT i eluates of weakly acid to alkaline nature; its only application having been to the eluate from sol ent partitioning procedure (Anton & Sayre 1972)

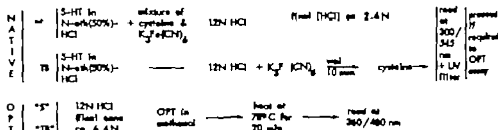
Conclusion: When assaying 5-HT the native assay i trong HCl has proved satisfactory for many requirements It is ery imple and rapid to perform with high sensitivity and when coupled with fairly simpl purification procedures the overall specificity is lso high The three derivativ assays for 5-HT ll provide greater sensiti ity; the most i tense fluorescenc being produced by the OPT assay which has probably been the one most widely applied when extra sensitivity is equired The other two assays so far less widely used may be valuabl for i cumstances where thei characteristi specifi ity i wanted Sensitivity can be of the order of 10^{-8} g 5-HT per cuvett ml when using the nativ assay i strong acid and 10^{-9} 10^{-10} g i the derivative assays

PRESENT PROCEDURE for the CONJOINT NATIVE and OPT-CONDENSATE ASSAY for 5-HYDROXYTRYPTAMINE

Introduction: The adaptation of two different fluorimetri assays for 5-HT to the N-ethanoli (50%)-HCl eluate from our new Dowex 50 purification procedure is described i Paper II

For assaying 5-HT i N -aqueous- HCl eluate obtained from CG 50 columns the nativ assay i trong acid with TB procedure incorporated i often preferred i our l boratories (Andén & Magnusson 1967) Therefore when using the Dowex 50 procedure from which similar eluate is obtained thi assay seemed good one to adapt since over number of years it has proved simpl rapid and reliabl i use However although its sensitivity i frequently adequate on some occasions greater sensiti ity would hav been appreciated Of the three more sensitiv assays for 5-HT the OPT assay was reported to give the most intense fluorescence and also it was performed i acid conditions Thus it seemed easier to adapt the OPT assay than the other two assays to the Dowex 50 eluate Preliminary tests showed that the OPT assay was suitabl whereas i the ninhydri assay considerabl interference was caused by thanol and material from the Dowex 50 resi There were suffi ient

similarities between the native and OPT assay procedures to encourage us to try and combine them i to a conjoi t procedure with common TB procedure such that both assays would be performed sequentially i the same tube By thi means we could benefit from the differing advantages of both assay procedures Since 5-HIAA and 5-HTP were separately purified in our Dowex 50 procedure the conjoint assay was also modified to suit thei respecti eluates



B 8 2 optima By using thanol we immedi tely obtained increased sensiti ity i the nativ assay for 5-HT the 50% thanol i the N HCl luate gi ing a 75% increase in fluorescence i tensity with littl effect on the OPT assay (for 5-HIAA, m thanol in the luate gav a 50% increase i the native assay nd also 25% increase i the OPT assay); the purity of the thanol (see section E 5 3) influenced TB values

Cysteine used i pr ferenc to ascorbic acid and the dispensing with UV light to complement th ferricyanide treatment both resulted in less quenching of nati fluorescenc Cysteine increased the tability of the 5-HT i ethanolic-HCl the effect being most marked i ery trong and hot acid as indicated by no decrease i native fluorescence under these conditions Thi will at least partly account for the previously reported increase in intensity and stability of fluorescence btained in the OPT assay i the presence of cystei e However cysteine may lso improve the conditions for the actual rea tion between 5-HT and OPT Ascorbic acid gav variable fluorescence and unacceptably high RBs i the OPT assay and could even decrease the stability of 5-HT in acid.

Ferric ions present in the concentrated HCl possibly the cause of bad batches of HCl i terfering i the nati e assay for 5-HT were also found to greatly decrease or prevent fluorescence being obtai ed i the OPT assay Furthermore cysteine which is used i the present and other published assays was found not to protect 5-HT against oxidation by F ++ ions i the concentrated HCl Consequently when using cysteine i the assays the HCl added in both nati e and OPT assays must be f from such impurities and to ascertain this a simpl test procedure was developed based on the ability of ascorbi acid to protect 5-HT against F ++ in the concentrated HCl

The OPT reagent obtained from Fluka and recrystallized was found to gi low blanks (as for histamine see section C 3.3) The optimum concentration of OPT in the reactant solution was 0 05 mg/ml which is i agreement with amounts commonly used.

Methanol was used as solvent for the OPT Its use was compared with xylene but we did not find the great variability with methanol which was experienced i the hi tamine assay by Yusem Delaney Lindberg & Fashing (1969)

Incubation t a temperature of 80°C for 20 minutes was found to be optimal The values shown in Paper II fig 1 were obtained following an incubation time of 20 minutes Thi time was chosen after preliminary experiment showed that for incubation temperatures of both 70° and 100°C fluorescence remained essentially unchanged between 10 and 20 minutes When incubating t the more commonly used temperature of 100°C fluorescence was more variabl and blanks were higher I the case of 5-HT and 5-HIAA assays this was probably partly due to evaporation of organi solvents However even with aqueous HCl which is more commonly used i analyses for 5-HT and 5-HIAA maximal fluorescence was lso obtained at 80°C

Fluorescence for 5-HTP was found to be already maximal t 40°C and emained essentially unchanged up to 80°C (see Paper II) Subsequently Tachiki & Aprison (1975) tudied i detail the assay for 5-HTP and found that by incubating t the lower temperature of 40°C for 60 minutes a ten fold increase in OPT concentration could be used and higher normality of HCl thi resulting i increased fluor escence and with lower and mor tabl blanks without the need for stabili ing agents which themsel es decreased fluorescence Apparently when incubating t higher temperatur lower OPT concentrations were necessary to avoid the formation of non-specifi fluorescence Thus I the conjoi t procedure for 5-HTP (Paper II tabl 1) the abov improvements could probably be incorpor ted to advantage: i after recording the nati fluorescenc the complet volume of 12 N HCl could be added prior to incubation the concentration of OPT could be increased and the sol tions incubated t 40°C for 60 minutes

However for 5-HT a temperature nearer 80°C for incubation may well prov to be optimal (as shown i Paper II); after incubat ing at only 60°C fluorescence was till increasing after 60 minutes (Tachiki & Aprison 1975) The suitability of incubation temperatures near 80°C for 5-HT has been confirmed by others (g Peuler & Passon 1973; Smith t al 1975)

blanks: Ferricyanide was chosen for oxidising 5-HT i the TB procedure When comparing ferricyanide and periodate both agents were found to oxidise 5-HT more effi iently i trong acid (littl oxidation occurring i HCl weaker than 1 N) and for these conditions UV-light i not required with ferricyanide However ferri yanide was found to be more specifi for 5-HT when applied to Dowex 50 eluates: it oxidised less material derived from resi and i addition periodate eacted mor with material derived from tissue Thi TB procedure utilizing ferricyanide and cysteine generally gav a Dowex 50 blank column error of less than 3 ng 5-HT per olumn and its accuracy was further checked using nervous tissue depl ted of 5-HT which after correction for the blank column error gave essentially ero values

B 8 4 specificity: The behaviour of number of 5-OR indoles in the native and OPT assays were compared to show th quantitati differences in the amount of fluorescence produced in th two assays g melatoni gave fluorescence three times that of 5-HT i the OPT assay and only half that in the nati assay Many of the 5-OR indoles which would i terfer i both assays for 5-HT including the m tabolites 5-HIAA and 5-HTP were removed by the Dowex 50 purification In f ct the only i terfering compounds found to be present i the 5-HT eluate wer bufotenine nd α-methyl 5-HT The conjoint nativ and OPT condensate assay was also used for measuring 5-HIAA nd 5-HTP after making small modifications appropri te to thei respective eluates from the same Dowex 50 column Most of the 5-OR indoles which were tested would be purified i to the same Dowex 50 eluate as 5-HIAA However thei presenc may be detectable by a discrepancy between the values obtained in the nativ and OPT assays (If further investigation wer to ascertai the identity of the interfering 5-OR indol i the assay of 5-HIAA then it may be possibl to qua tify both indoles i the same conjoi t native and OPT condensate assay by means of imultaneous equations: e g in pure solutions we wer ble to differenti lly quantify 5-HIAA and 5-hydroxytryptophol in mi tures; however if the conjoi t assay was to prov useful i establishing the presence of an i terfering 5-OR indol then we would probably prefer to choose purification procedure i which these compounds were separated prior to assay)

B 8.5 sensiti ity: In the native assay increased fluorescence was obtained by the presence of thanol the use of cysteine instead of ascorbic cid and th omission of UV light from the ferricyanide treatment Each unit above the RB was equivalent to ca 0 95 ng 5-HT per tube By contrast using the much greater fluorescence i tensity obtained i the OPT condensate assay 1 meter unit was equivalent to 0 07 ng 5-HT per tube and since the reproducibility and accuracy of the TB procedure was high smaller amounts of 5-HT could be quantified with greater eproducibility The sensiti ity of the OPT assay was not decreased by the incorporation of the native assay The assay was shown to have a linear relation between fluorescence and concentration from 1 5000 ng 5-HT and the eproducibility of the assay is such that 10 ng 5-HT per column could be qua tified

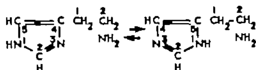
B 8 6 Conclusion: Th tention of the nativ assay for 5-HT permits greater continuity of data to be mai tained using this type of assay which over a number of years has been found to be simpl and reliable and whi h remains satisfactory for many purposes Its incorporation i to a conjoi t procedure i which the OPT assay can be sequentially performed i the same tube with a blank procedure common to both assays provides an opportunity to gai experience and confidence with the OPT assay by a di ect comparison of results. Then on those occasions when th native fluorescenc is difficult to ead the procedure may be continued to utilize the extra sensitivity of the OPT assay Furthermore quantitativ differences in interference by other indoles i the two component assays provide doubl check on

specifi ity; discrepancies between th two assays have been reported previously (Baumgarten Evetts Holman Iversen Vogt & Wilson 1972)

The conjoi t assay procedur has been presented i forms suitabl for application to 5-HT 5-HTP and 5-HIAA i thei appropriate eluates from the new Dowex 50 purification procedure This i our preferred combination of purification and assay procedures for 5-HT when measurements are also required of its metabolites or other amines For measurements of only 5-HT we still prefer to use our simpler and more rapid CG 50 purification procedure (Andén & Magnusson 1967) Moreover we hav been abl to combine its advantages with those of the onjoi t native and OPT-condensate assay applying the same assay schedul to the N-aqueous-HCl eluate The small eluate volume (3.5 ml) i conjunction with the OPT assay provides very specific and sensitiv overall procedure for d termining 5-HT

Section C FLUORIMETRIC ASSAY of HISTAMINE

C 1 Introduction: For reviews of the fluorimetri assay of Hm see e.g McI tire (1966) Udenfriend (1969) The structure of histamine (Hm) 4(or 5)-(2-amino ethyl) imidazol and its chemical properties hav been revi wed by Jones (1966) (see lso Ganellin 1973) Hm lacks useful light absorption or emission properties unlike the ther amines under consideration and consequently there is no assay based on native fluorescenc Th only fluorimetric assay for Hm which has been widely used is that based on its ea tion with OPT to form a fluorescent derivati



C 2 ORTHOPHTHALDIALDEHYDE CONDENSATE ASSAY for Hm

Hm i trong NaOH sol tion + OPT pH ca 12 7	condensation temperature/time dependent	+	Acid to give pH ca 2.5	room ⟶ temp	read at 350/450 nm

C 2.1 optima: The fluorimetri assay for Hm based on its condensation with OPT i trong alkaline solution followed by acidification to produc the fluorophore was introduced by Shore Burkhalter and Cohn (1959) who applied it to tissues (for brai tissue see C 3.7) Subsequent alterations hav given greater reproducibility and small increases in sensitivity However a ecent detailed investigation of the assay has resulted i greatly increased sensiti ity and specificity (Håkanson Rönnberg & Sjölund 1972; Håkanson & Rönnberg 1974)

The lkaline pH at which condensation of Hm with OPT occurs optimally was found to be ca 12.7 by Håkanson and co-workers (1972) The amount of NaOH required depends on the natur of the luate and in most published assays a pH between 12.5 and 13.5 is achieved The purity of the OPT i important (see g Yusem t al 1969) and most laboratories ecrystalliz the manufacturer s product As a sol ent for OPT methanol i generally employed lthough Yusem et al (1969) were unable to obtai results as reproducibl as with xylene The concentration of OPT used i the assay is particularly important: i e cess OPT may additionally eact with other substances

to produce non-specific fluorescence the i terference of which i not easily recognised because f the inadequacy of th TB procedure (see C 2 2) Th lower concentration of OPT of 0 1% instead of 1% was added in the assay by Noah & Brand (1961) The effects of this change wer studied by Redlich & Glick (1965) who found that the lower concentration of OPT gav greater reproducibility and tability of fluorescence i addition to much greater decrease of fluorescenc in the S as compared with the St The small loss of Hm fluorescence was later avoided by slightly increasing the concentration of added OPT to 0 2% (≈ 0 07 mg/ml i the reactant solution) and thi lso gav a 50% decrease i RB fluorescence when compared with a 1% sol tion of OPT (Håkanson t l 1972) EDTA has been used i the alkaline sol tion (Kremzner & Pfeiffer 1966) to help prevent the decreased yi ld of fluorescence induced by many cations (Kremzner 1966; Håkanson t l 1972) The reaction has until recently (see below) always been performed t about room temperature allowing ca 4 minutes (Shore et al 1959) sometimes i water bath to gai increased reproducibility (e g Yusem t al 1969)

Acidification (to pH of ca 0.8) was found by Shore et al (1959) to tabilise the fluorophore which is rather i bil i alkaline sol tion Increased sensitivity was obtained following the observation that fluorescence intensity increased rapidly up to pH 2 and then emained constant to beyond pH 4 (Kremzner & Wilson 1961) the actual pH range varying slightly depending on the acid used (Håkanson et l 1972) Sulphuric acid gives a slightly higher fluorescence i tensity than other acids extending to pH 6 probably due to *the slight potenti tion of fluorescence by SO_4^{--} ions as compared* with the quenching caused by most ther anions (Håkanson t l 1972) However phosphoric acid with its good buffering capacity has been most commonly used to gi a pH between 2 and 3 (Kremzner & Wilson 1961); ther acids used include itri acid (A ton & Sayre 1969) and HCl (Thompson 1961)

Fluorescence i tensity i a improved Hm assay has been more than doubled as a result of the detailed studies recently made by *Håkanson Rönnberg and Sjöland Perhaps thei most surprising finding* concerns the temperature optimal for the Hm-OPT condensation considering that for over 10 years condensation for ca 4 minutes at room temperature has been so widely adopted Fluorescence was increased by ca 35% using a condensation t 0°C for 40 minutes (Håkanson t al 1972) An additional 36% increase i fluorescence was obtained from the reaction t 20°C for 10 hours this being the optimal temperature for the Hm OPT condensation determined by Håkanson & Rönnberg (1974); *lower RB were lso obtained Furthermore itrogen* to gas the reaction mixture was used i these tudies to obtai an additional 40% increase i fluorescence; thi following thei observation that the condensation of Hm with OPT was adversely affected by the presence of oxygen. Recently the increased sensitivity gained by adopting some of these modifications (incubation t 0°C under N_2) was confirmed but retention of conventional assay was recommended on the grounds of decreased reproducibility (Braidwood & Owen 1974) This seems somewhat precipitous since only one factor e g -gassing

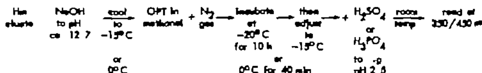
with N_2 may hav led to the increased variability whereas several factor contribute to the increased sensiti ity of the improved assay

C 2.2 blanks: I the commonly used reversed blank procedure ("B-rev") included i the original procedur of Shore et al (1959) OPT is not added until after the final acidification (I some schedules OPT is entirely omitted) However it would appear that materials which react with OPT i lkaline solution to form non-specific fluorescence frequently do not do so i acid solution Thus the TB-rev" is not an accurate quantitation of non-specific fluorescence and being too low its use leads to f lse over estimations of Hm values: for example even with pure solutions RB-rev can be lower than the RB (Redlich & Gil k 1965; see also C 3.4 below) In this respect a TB procedure is equired i which the OPT i present in alkaline sol tion but can only rea t with those substances contributing non-specifi fluorescence

Automated ersions of the OPT condensate assay for Hm (condensation at room temperature) hav been developed (Evans Lewis & Thomson 1973; Siraganian 1974)

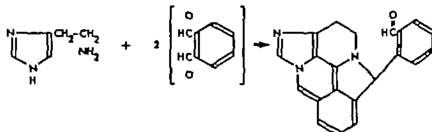
Additional aspects of the assay are considered below (section C 3)

Utilising the improvements discussed abov an assay i which great sensiti ity is equired for Hm could hav th following format:



In the TB-rev procedure the addition of OPT is postponed until after the final acidification

C 2.3 chemistry: Rönnberg Cederlund & Håkanson (1977) isolated the fluorophore formed from the condensation product of one molecule of Hm with 2 molecules of OPT to yield thi polycycli compound The



greater part of the reaction occurred i the alkaline solution (pH 12 13) the sequence of events suggested by Rönnberg et al (1977) (see also Shore t al 1959) invol ing the condensation of one molecul of Hm with one molecule of OPT and proceeding through Schiff base formation and i tramolecular cyclization to an imidazotetrahydropyridene derivativ which condenses with a second molecul of OPT followed by further cyclization to form a labil compound Acidification (to pH 2 4) induces a final dehydration to yield a very tabl product highly fluorescent i the protonated form.

specificity: The OPT-condensate assay for Hm as commonly used with incubation at room temperature has a high degree of specifi ity Substitution of the imidazol-ethylamine structure at the 1 (ring) or N (side chai) positions prevents fluorescenc (the 2-side chai substitution as in α-methylhi tamine not having been checked) thus catabolites of Hm including 1-methylhistamine 1 4-methylimidazoleaceti id imidazoleacetic acid N-methylhistamine and N-acetylhistamine do not interfere (Shore t 1 1959) The only compounds which may giv considerabl equimolar i terference or other imidazolethylamine derivatives unsubstituted in these positions. Hi tidine the precursor of Hm is probably the most important of these gi ing ca 15% i terference; other examples or histidinol (the thanol precursor of Hd) (ca 26% i terference) nd various histidyl peptides and esters of Hd (Shore t al 1959; Graham Scarpelli i Hubka & Lowry 1968; Boissier t al 1969; Gerber 1970) Som ther compounds less structurally el ted gi small amounts of i terference (see also Kremzner & Wilson 1961; Kremzner & Pf iffer 1966; Ambrose Crimm Burton Paullin & Ross 1969) but the majority generally occur i tissues i amounts not affecting the Hm determination and most are non-bases and thus easily separated from Hm However some of these compounds can be present i amounts greatly i excess of Hm such that thei weak molar fluor escence can nevertheless cause significant i terference i the Hm assay Important examples being NH_3 (Shore t al 1959) and spermidine. Thus interference by Spd is ca 4% (Michaelson & Coffman 1967; Boissier t 1 1969) but for a number of years the large amounts of Spd present i brai up to 1000 times that of Hm have presented particular problems for the assay of Hm i this tissue (Kremzner & Pfeiffer 1966) (see also section C 3.7) Slight modifications of the assay hav produced separate assays for Hd (e.g Ambrose et al 1969; Gerber 1970) and Spd (Kremzner 1966)

However increased specificity as well as sensitivity is achieved when performing the assay under the more optimal conditions ecently described Hm fluorescence intensity was greatly increased with concomitant decrease i fluorescence from Hd (4%) Spd (0.03%) and histidyl derivatives (Håkanson & Rönnberg 1974) The reasons for the increased specificity become more obvious when comparing the conditions used i the assay for Hm with those for Hd and Spd when separate optimi ed OPT-condensate assays for these compounds were established with consequent gains i specifi ity and sensitivity Thus optimal conditions were for Hm condensation with OPT at pH 12.4 – 12.7 under N_2 at 20°C for 10 h followed by acidification to

pH 2 3 with fluorescence maximal at 350/450 nm; for Hd condensation at pH 11 2 11 5 without N_2 at +40°C for 10 min and without final acidification fluorescence being maximal at 360/440 nm (the arious optimal combinations of conditions were also established for histidinol and a number of histidyl dipeptides) (Håkanson Rönnberg & Sjölund 1974); for Spd, condensation t pH 11 0 11 6 without N_2 at 100°C for 2 mi and with final acidification to pH 3.5 fluorescence being maximal at 350/400 nm (Håkanson & Rönnberg 1973)

Assays for a number of other compounds of biological i terest have been based on their reaction with OPT g 5-HT and other 3-alkyl 5-OR indoles (in hot ery strong ca 6.5N HCl) (Maickel & Miller 1966; see also section B 4); metaraminol and other primary m-hydroxy-phenyl ethylamines (at pH 9 followed by acidification) (Shor & Alpers 1964); (also agmatine i strong alkali without cidification Cohn & Shore 1961) Obviously umber of factors ar important for obtaining maximal fluorescence from compounds i OPT condensate assays: these include the pH presence or absenc of O_2 and combination of temperatur and time used i the incubation for condensation to occur and the lkaline or acid pH and wavelengths t which maximal fluorescence is exhibited Thus although OPT eacts with a large number of compounds to produc fluorophores provided that reaction conditions are carefully optimized for each compound or group of compounds sensiti assays are obtained with a high degree of specificity

The choic of purification procedure for Hm depends largely on which i terfering compounds ar likely to occur: e g NH_3 i urine; Spd i brai and blood; Hd in tomach or after admi istration or induction. The assay has been used for measuring Hm i various biological materials using for example Duolite ES 63 for brai (Michaelson t al 1968) Decalso for blood (Graham et al 1968) Dowex 50 for brai (Atack 1971) and combined with solvent partitioning for blood liver and gut (Lorenz et l 1970 1972) The assay has been applied to eluates from most ommonly used purification procedures lthough not all effecti ely separate the important interfering compounds from Hm (see section A)

C 2.4 sensitivity: The Hm OPT condensation t 20°C under N_2 gave a sufficiently sensitive and specific assay for direct application to unpurified tomach extracts (Håkanson & Rönnberg 1974); even though in thei ecommended procedure only small amounts of extract i relation to assay reagents (0.1 2.5 ml) were used However for assaying tissues with relatively low Hm concentration g brain continued use would be mad of a purification procedure which concentrated the amine i to small olume and removed materi l which may gi high blanks and quenching Much higher atios of luate to assay reagents can then be used as i most other published assays to gain increased sensitivity This would allow full advantage to be taken of the improvements i the assay to gi e ery sensitive and specific overall procedure for quantifying Hm. Sensiti ity i th assay can be of the order of 10^{-9} g Hm per cuvette ml with condensation at room temperature increasing to 10^{-10} g Hm per cuv tte ml with condensation t 20°C

C 3 PRESENT PROCEDURE for the FLUORIMETRIC ASSAY of HISTAMINE:

C 3.1 Introduction: T assay Hm I a 2N-aqueous-HCl eluate after its purification on a Dowex 50 column the OPT-condensate procedure of Shore et al (1959) modified by Kremzner & Wilson (1961) was adopted by Green & Erickson (1964) The results they obtained for Hm I rat brai are in good agreement with presently accepted values (see C 3.7) Therefore inc I our Dowe 50 procedure Hm is also purified I t 2N-aqueous-HCl eluate (see Paper I) we felt justified I using the assay without a detailed study Practical detail and comments on the procedur which we hav used for assaying Hm I the Dowex 50 luate are given

C 3.2 Hm assay: After extraction from tissue and purification on the Dowex 50 columns Hm was eluted with 4 5 ml of 2N-aqueous-HCl and ca 0.07 ml 6 0 N distilled HCl was added to bring the eluate normality to 2.0 N I compensation for a dil tion from residue of N-aqueous-HCl I the column procedure; the eluates then being tored at 20°C until required for assay (for full detail see Paper I)

Histamine Assay	St*	RB	RB -rev	S	ISt*	TB -rev
	ml	ml	ml	ml	ml	ml
H_2O	—	0.05	0 05	0 05	—	0.05
2N-aqueous-HCl	0.8	0 8	0 8	—	—	—
Eluate: (2N-aqueous-HCl)	—	—	—	0.8	0 8	0 8
Hm(base) e.g 1 μg/ml	0 05	—	—	—	0.05	—
4.6N N OH†	0.4	0.4	0 4	0 4	0.4	0.4
4% EDTA	0.05	0.05	0 05	0 05	0.05	0 05
cool for 5 ml at 20 25°C In water bath						
0 3% OPT I methanol (99%)†	0 05	0 05	—	0 05	0 05	—
wait for 4 ml at 20 25°C						
2.0M H_3PO_4†	0.1	0 1	0 1	0 1	0 1	0 1
0 3% OPT I methanol (99%)†	—	—	0.05	—	—	0.05

OPT-condensat fluorescence was read withi 10 60 min t 350/450 nm.
Final pH of 11 solutions was then checked to be ca. 2.3 (range 2 3)
† Ref the are normally used for these deliveries.
Final volume = 1 45 ml (contai ing 0 8 ml portions of 4 56 ml eluate)

The eluates were brought to room temperature and thoroughly shaken before use Up to 18 luates could be conveniently assayed at one time From each eluate three equal portions of up to 0.8 ml with smaller volumes being made up to 0.8 ml with pure

2N HCl were used for S ISt nd reversed tissue blank (TB-rev) (although TB-rev wer often not prepa ed see below) The equivalent eluate from the blank olumn was used for PS P ISt and PB-rev These were run I paroll l with three St two RB and RB- ev The compl te assay procedure was performed I silica or pyrex glass tubes the contents of th tubes being thoroughly mixed on a ortex mixer after each step Fluorescence was ecorded using an Aminco-Bowman spectrophotofluorimeter fitted with an on-axis enon lamp P 21 photomultiplier tube and and slit arrangement no 4 The assay schedule is tabul ted

C 3 3 optima: Th alkaline pH was found to be optimal at 12 9 However N OH was used to giv a pH of 13.1 si ce despite 5% loss of l tensity fluorescence was more reproducible because small errors l the volumes of the strong HCl and the somewhat iscous NaOH had less effect (as illustrated for the DA assay l Paper III fig 1) The use of a rapid repetitive device (RePette see Paper III) for forcibly delivering the NaOH solution ided accuracy The mi tur was cooled to 20 25°C befor adding the OPT because of th temperature/ /time dependenc f th reaction (the advantage of using lower temperatures and N_2 had not been published when thi assay schedul was prepared)

OPT obtained from Fluka (see Paper II) nd recrystallized esulted l imilar fluorescence but lower blanks than with ther products tested OPT was recrystalli ed from anhydrous (using Na_2SO_4) petroleum ether (BP 60 71°C) dried with tream of N_2 gas and tored for up to 1 year at 20°C under N_2 small amounts being placed l several snap-topped small dark glass bottles A bottl was warmed to slightly bov room temperature befor opening The OPT was dissol ed l 99% methanol little vari tion in Hm fluorescence was found whether methanol or xylene was used but the latter was un pleasant to handl Th OPT sol tion was freshly prepared before each Hm assay since there was a tendency for blanks to become more variabl if older solutions were used (any unused solution could be stored l small dark-glass bottl t 4°C for up to 1 week for use in the 5-hydroxyindole assays see Paper II since blanks wer found to be less affected l these assays) To decrease non-specifi fluorescence the mi imal OPT concentration was determined which gav maximal net fluorescenc (*St RB) for a concentration of Hm (e g 250 ng) towards the upper limit normally measured withi the range of linearity To check that this amount of OPT eacts preferentially with Hm the assay was first applied to blank column eluates and then to tissue derived eluates: Hm recoveries (*ISt St) were reproducibl and often close to 100%. Optimal esults were obtained with 0.05 ml 0 2 0.4% OPT and a 0 3% solution (gi ing 0 12 mg/ml reactant solution) l used

To obtai a final pH abov 2 acids with some buffering capacity were tested l phosphoric citric and acetic acids rather than sulphuric and hydrochloric acids. Phosphori acid gave higher fluorescence than ac tic acid and imila fluorescence but lower blanks than citric acid fluorescence being maximal and more reproducibl

from luates when adjusting to a final pH of 2 3 The final pH was measured using a combined microele trode as simple and rapid check for errors I the olumes of HCl and NaOH used

C 3.4 blanks: In this assay procedur we used S PS rather than S TB-rev for routinely orrecting sampl eadings This was because the reverse blank was found to be too inaccurate: non-specific material derived from the purification procedure and tissue which reacts with OPT at an alkaline pH did not appear to do so at an acid pH Thus the TB rev and PB-rev did not differ significa tly from the RB and the RB-rev* was usually 1 or 2 units lower; whereas the PS* was usually one to two times higher than the RB being equivalent to blank column error of ca 3-9 ng Hm per column (see C 3.6) However t least an indication of the amount of non-specifi fluorescenc ontributed by eagents and purification procedure can be gained (*PS) and used to orrect the S Furthermore the effect of tissue-deri ed materi l may well be small due to th external washes used fter placing th extract on th column (especially since in the 5-HT assay with accurate bl nks we obtai TB only slightly higher -ca 25%- than PBs ; and Hm l el ted afte 5-HT)

The perfect TB procedur would measur non-specifi fluorescen resulting from condensation with OPT t th lkaline pH Such blank procedure could involves selecti destruction of Hm as used i oxidi ed blanks g for 5-HT (see B 3 2); selecti blocking of the Hm OPT condensation; or selecti destruction of the Hm fluorophor The formation of the Hm fluorophor (but not the Spd fluorophor) has been found to be prevented by the presence of formaldehyde (Håkanson t l 1972) but i ery preliminary experiments we found it also prevented th formation of non-specifi fluor escence from resi mate ial, i the PB was till lower than the *PS

Thus i the bsence of easonably accurate TB procedur consideration may be given to choosing purification procedure whi h removes as much tissue deri ed material as possibl even though it contributes som i terfering material rather than procedure which contributes no or littl i terferenc itself but llows much tissue-deri ed material i to the luate because the contribution of non-specifi fluorescence by the purification procedur i quantifi-bl (*PS RB) Analysi of brai tissue depl ted t its Hm content would provide valuabl check on the amount of non-specifi fluor escence ontributed by the tissue but such depletion of Hm is more difficult to achieve than for the monoamines

C 3.5 specifi ity Compounds whi h have been reported to be abl to i ter fere i the Hm OPT-condensate assay were mentioned abov (C 2.3) Some of these compounds we hav shown to be separated from Hm including Hd and Spd and of the remainder most are non-bases and are very likely to be separated from Hm i the Dowex 50 procedure for purifying Hm (Paper I) prior to its assay

C 3.6 sensitivity: The following readings for relative fluorescence intensity are typical of those obtained in an assay of Hm extra ted from ca 2.5 g normal mouse brain

St* 50 ng	RB	RB -rev	PS	P* ISt	PB -rev	S*	ISt*	TB -rev
0 302	0 008	0 006	0 012	0 300	0 007	0.110	0 400	0 007

From these eadings it may be calculated that one meter unit above the RB (x 0.001 scal) is equivalent to 0 17 ng Hm per tube and twice the RB is equivalent to 1 4 ng Hm; also the PS is for eluates from well washed resin olumns generally one to two times the RB these data being imilar to those obtained i the 5-HT and DA assays (Papers II and III) However the TB procedure is very inaccurate and generally we correct the S by PS other than by TB-rev the blank column error" (here being 3 9 ng and generally 3 9 ng Hm per column) is then automatically accounted for The ISt% here being 98 7% generally ranged from 89 101% (data taken over several weeks) In the example given S PS" is equivalent to 16 7 ng per tube i i the 0 8 ml portion of a 4 56 ml luate which is equivalent to 95.0 ng Hm per column which i turn i deri ed from 2.00 g mouse brai placed o the column (calcul ted after allowing for losses when once extracting 2.44 g mouse brai see Papers I and III); thus the Hm con entration i thi normal mouse brain is 47 5 ng per g

The assay (OPT condensation at +20 25°C) is considered to be useful for measuring Hm i amounts as low as 10 ng Hm per column (10 ng per column into a 4 56 ml eluate = ca. 2 ng per 0 8 ml portion taken for assay; and 1 g rat br i would provide ca 50 ng per column) Nevertheless the doubling of sensitivity reported to be gained i the assay by performing the condensation with OPT under N_2 at 20°C will be appreciated The values we hav btained for Hm i several different tissues are i good agreement with other published data and this is especi lly important with respect to alues for Hm i brai

Hi tamine concentrations of ca 48 ng per g i normal mouse brai which we obtai when using Dowex 50 purification together with fluorimetri assay (see g. Atack 1971) may be compared with values of 60 ng/g obtained using organi solvent partitioning and fluorimetri assay (Anton & Sayr 1969) 60 ng/g by bioassay (Adam H personal communication 1967 i Green 1970) and 49 ng/g by an enzymatic isotope derivati e procedure (Taylor & Snyder 1972)

C 3.7 histamine i br in: In 1959 th OPT condensate assay for measuring Hm i several different tissues was introduced by Shore t al for rat brai Hm values of 200 400 ng per g were obtained i good agreement with bioassay In 1963 this agreement was shown to be fortuitous since both assays suffered from i terference (Carlini & Green J. 1963) I 1964 Green H & Erickson used a Dowex 50 purification procedure t separate Hm from materials i brai which interfered

I the OPT assay but the procedure was unfavourably considered (see Green J 1964) this being somewhat unfortunate since this Dowex 50 procedure was the first to adequately purify rat br I Hm for the OPT-condensate assay gi ing a value of 52 ng per g whi h is withi the presently accepted range

I 1966 Kremzner & Pfeiffer identified Spd as the major contaminant I the fluorim tri assay of brai Hm and used a phosphoryl ted cellulose column for its separation Only with the development of improved forms of this type of purification procedur which are rarely used for the monoamines (see section A) did the fluorimetric assay of Hm begin to become widely used for th analysis of Hm I brai

However by thi time fluorimetric assays for the monoamines had been i routine use for more than 8 years th DHI assay for DA being I troduced by Carlsson & Waldeck I 1958. Thei rel ti simplicity had led to a vast expansion I research I to the ol of monoamines I the CNS which unfortunately largely by-passed Hm although the enzymati isotope-derivati technique (Snyder et al 1966) provided much useful information (see Snyder & Taylor 1972)

The facility which we have I troduced of luting Hm from the same Dowex 50 column but separated from the monoamines and Hd and Spd (Paper I) coupled with the fluorimetri assay will hopefully encourage the more frequent determination of Hm I some of the many experiments which are prima ily designed for tudying the rol of the monoamines W hav al eady mad some I teresting findings by taking advantage of thi f ility

Thus whil using reserpine to depl te existing DA tores and Ro 4 4602 to prevent new DA formation as established techniques I tudies of DA turnover and receptor activity we took the opportunity to determine the effect of these drugs on the Hm I mouse brai (Atack 1971) The Hm concentration I mouse brai of 48 ng per g was found to be decreased both after reserpine admi istration (by 11%) and after Ro 4 4602 (by 34%) and I combination thei effects were additi (44%) These results may be an indication that som Hm may be tored in granules I form whi h is released by reserpine The quantitative effect of eserpine on brai Hm shows considerabl species variation (Green & Eri kson 1964; Adam & Hye 1966) but for mouse brai our findings of decrease were substanti ted by Taylor & Snyder (1972) Ro 4 4602 induced decrease of Hm I experiments wher its intended use was to block the synthesi of monoamines by inhibiting the aromati amino acid decarboxylase Hd can be decarboxylated to Hm by both thi enzyme and by specifi hi tidine decarboxylase but which enzyme predominates may vary for different brai areas and for different species It may be that Ro 4 4602 also blocks the specifi decarboxylase (see Snyder & Taylor 1972) Thus when using drugs to manipul te the putative transmitter monoamines it may be remembered that some of them will also be effecting Hm.

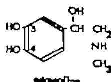
In another investigation we obtained esults which provided further evidenc of Hm being a putativ transmitter Hm has

satisfied fewer criteria which characterise a neurotransmittor than do the monoamines NA DA and 5-HT A neurotransmittor should be released upon depolarization of nervous tissue Th release of the monoamines from depolarized nerve tissue is well established and we have used this fact in an experimental model with slices of mouse brain hypothalamus I itro I which depolarization was induced by increasing the K^+ ion concentration to determine how the release of the monoamines was modified by various drugs. Since th release of Hm from depolari ed nerve tissue had not previously been demonstrated we took the opportunity of measuring Hm I some of the experiments and determined a elease (Atack & Carlsson 1972) In an independent tudy using a different assay technique for measuring Hm Snyder & Taylor (1972) also showed that Hm is released by increased K^+ ion concentration and furthermore this release resembled that of NA not 5-HT I equi ing the presence of Ca^{++} ions.

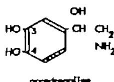
C 3.8 conclusions Our I terest I Hm is mai ly oncerned with its behaviour in comparison with the monoamines The Dowex 50 procedur I which Hm and the monoamines f om singl tissue extract are purified I to separat eluates coupled with the OPT condensate assay for Hm gives an overall procedure with high degree of sensiti ity and specificity especially with espect to the removal of Hd and Spd For measuring only Hm I termedi tely acidi (phosphonic) cation exchange esins used prior to the assay also provide similar sensiti ity and specificity However for our convenience the same Dowex 50 col umns which ar used for successively el ting several biogeni amines can also be used for el ting only Hm I simpl rapid purifi ation procedure in which the high sensiti ity and specificity are etained (see Paper I)

Section D FLUORIMETRIC ASSAY of ADRENALINE and NORADRENALINE

D 1 Introduction: For reviews of fluorimetri assays used for A and NA see e g von Euler (1961); Udenf iend (1962 1969); Häggendal (1966); Weil-Malherbe (1968 1971) and Sharman (1971) The tructure of adrenaline (A) (epinephrine) I e 1 (3 4-dihydroxyphenyl)-2 (methylamino)ethanol; and of noradrenaline (NA) (norepinephrine) I e. 1 (3 4-dihydroxyphenyl)-2 -aminoethanol; and thei hemical properties hav been reviewed by on Euler (1956 1961)



adrenaline



noradrenaline

D 1 1 catecholamines general The catecholamines (CA) A NA and DA can ll be measured using fluorimetri assays based on the same three principles: nativ fluorescen condensation with thylenediamine (EDA) and formation of a hydroxyindol (HI) The nati assay has only a ery limited use The EDA and HI assays both had considerabl usage I the 1950 but I subsequent years the latter gained ascendancy

Although assay conditions may sometimes be adjusted to favour maximal fluorescence from any one of the CAs the others usually to greater or lesser extent give I terference This problem I compounded by the fact that I many routine purification procedures the three CAs are not separated A large number of modifications particularly of the HI assays hav been mad I an effort to 1) increase the ccuracy of quantification of the indi idual CAs; 2) produce even small increases I sensiti ity Ince tudies of many of th functions of these CAs have tended to requi thei measurement I amounts ery close to the limits of sensitivity of the fluorimetri assays; and 3) to overcome local environmental problems which may result from small unrecognised differences I procedur I different laboratories effecting the somewhat complex chemistry of the eactions; these probably accounting for high percentage of the numerous modifications of these assays (see lso Weil Malherbe 1968).

The separation of DA from A plus NA I achieved by only one of the routine types of purification procedure and I addition the most specifi of the fluorimetri assays tend to be for DA. Therefore the assays for DA re considered separately (see section E)

D 1 2 A and NA: In contrast A and NA are extremely difficult to separate from each other in most routine purification procedures and it is also proving difficult to develop reproducibl assays for each of these amines with negligable interference by the other In consequence assays for A nd NA ar generally considered together However assay conditions can be modified to favour maximal fluorescence from either A or NA so that when both amines ar present i the same luate they can be quantitati ely differenti ted by recording fluorescence under two different sets of conditions

The simplest approach is to use a singl assay and discriminate on the basi of small differences i wavelength characteristics t which maximal fluorescence is obtained (wavelength maxima) This approach has the advantage that no di ision of the eluate for two assays is required but maximum differentiation is generally achieved t non-optimal wavelengths with consequent loss of sensiti ity nd greater variability and i general thi approach i only suitabl where the amounts of A nd NA do not differ by more than a fa tor of 10

Another approach is to use a two-component assay procedure and i t least one and pr ferably both of the assays the conditions are adjusted to obtai maximum fluorescenc f om one amine with mi imum fluorescence by th ther Thi has th advantage of being abl to measur each amine using near optimal conditions to achiev maximal sensitivity with high reproducibility and gives the highest discrimination between A and NA Thus it is much more suitabl when the two amines are present i greatly disproportionate amounts However the disadvantages ar the need to sub-di ide the luate the time needed to do two assays and the summation of thei ariabilities

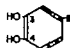
D 2 NATIVE FLUORESCENCE of A and NA I ACID


A or NA I 0 1 N HCl ——— Read t 285/325 nm

Thi simpl assay for A and NA i based on the nativ fluorescenc of CAs at 285/325 nm which i optimal t pH 1 (Duggan Bowman Brod & Udenfriend 1957) It has been used for measuring total CAs i tissues rich i CAs g the adrenal gland (Bertler et al 1958) However for most applications this assay is far too insensitive *and lacking i specificity compared with the THI and EDA assays* Moreover these l tter types f assay were l eady well established when i the mid-1950 s the introduction of the spectrophotofluorimeter made possible the use of wavelengths i the ultra-viol t range such that the nati fluorescence of CAs could be recognised (Bowman t al 1955) The nativ fluorescenc of CAs is weak; also blanks tend to be high because f the close proximity of activation and fluorescence wavelengths

Apart from the CAs large number of ther compounds exhibit native fluorescence at the same or similar wavelengths not

only most catechol compounds and 3-O-methylated derivati es thereby including t least the majority of CA metabolites and also many of thei pharmacological analogues but also many compounds incorporating a phenoli tructure (Duggan t al 1957 Udenfriend 1959 1962; Aminco eprint 1960) Furthermore the weak CA fluorescence can be i terfered with by the overlapping much more i tense fluorescence of some other groups of compounds e g 5-HT and ther indoles this point should be borne i mind when using the nati fluorescenc as simpl nd rapid means of quantification i fractionation studies to establish the distribution of catechols and thei separation from indoles g CAs from 5-HT in purification procedures (see Paper I)



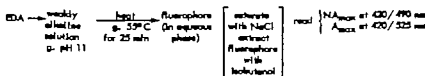


R alkyl side-chain

D 3 ETHYLENEDIAMINE CONDENSATE ASSAY for A and NA:

D 3.1 Introduction: For reviews of these assays see g Weil Malherbe (1959 1971) Manger Wakim & Bollman (1959) von Euler (1961) and Udenfriend (1962) Th assay i based on the autoxidation of CA to its quinone derivativ and subsequent condensation with EDA to form fluorophore the complete reaction occurring i the same weakly lkaline sol tion. The assay was first described for A i pure sol tions by Natelson Lugovoy & Pincus (1949) and was subsequently developed by Weil Malherbe & Bone (1952) for measurement of A lik substances i blood plasma the A and NA components i ter being differentiated by taking readings t two sets of wavelengths (Weil Malherbe & Bon 1953; Persky & Roston 1953)

NA or A in eluate



D 3.2 optima i thi impl procedur the autoxidation and subsequent condensation take place i the same solution The rati of EDA CA i important for optimal fluorophore formation (e g Burn & Field 1956; see Weil Malherbe 1959) However the free EDA base has the additional function of producing the pH for the reaction values of g. pH 10 4 11 and 12 having been given as optimal (Weil Malherbe & Bone 1952; Burn & Field 1956; Erne & Canbäck 1955). Thus to achieve both the optimal amount of EDA and the appropri te pH a mi ture of EDA base and EDA HCl may be used the proportions depending on the nature of the eluate (e.g Bone 1953; Erne & Canbäck 1955) Normal mi ing of the incubation mi tur may provide sufficient aeration for autoxidation (see Weil Malherbe 1959) although the addition of an oxidising agent g. O_2 may be found necessary

(e g Burn & Fi ld 1956; Nadea & Sobolewski 1959) Other modifications and comments rel ting to the assay (see also discussion by Weil-Malherbe 1959) include the use of different temperatures and periods of incubation (e g Bone 1953; Erne & Canbäck 1955; Burn & Field 1956; Kägi Burger & Giger 1957) and molybdate as a catalyst in the NA assay (Erne & Canbäck 1955) The formation of CA molybdate complexes as preliminary step in the assay may hav advantages (Nadeau & Sobolewski 1959) they were eliably oxidised by dissolved I I contrast to the free CAs; amine fluorescence was increased and blank fluorescence decreased; and ther was decreased i terference by metalli ions e g. Al^{+++} ions particularly when assaying A (Valk & Pri 1956 Mangan & Mason 1957 Nadeau Joly & Sobolewski 1958). The possibility of i terference by g Al ions i EDA assays may be checked when first using lumina for purification by comparing PS and "ISt for a blank purification eluate with external St"

The fluorophores formed from A nd NA i queous solution are generally extracted i to isobutanol This results in a considerabl increase i fluorescenc i tensity in addition to concentr ing the fluorophore i to a smaller volume; lso small wavelength shifts occur to 420/490 nm for NA-max and 420/525 nm for A max (see Laverty & Sharman 1965)

If the CAs are ac tylated prior to the condensation with EDA fluorescence may be further increased by ca two-fold for the NA product and six-fold for the A product but a disadvantag is that wavelength maxima of A and NA become identical (420/490 nm) (Laverty & Sharman 1965; see al Crawford & Yates 1970) Assay of the acetyl ted derivatives has been more widely used for DA (see section E 3) than for A and NA

The NA fluorophore is considerably les stable than that of A i the presence of light (Aronow & Howard 1955; V lk & P ice 1956; Goldfien & Karler 1958; Harley-Mason & Laird 1959) and to inc ease its stability darkened (low-actinic) glasswar has been used throughout the assay (Weil Malherbe 1961 1971)

An automated ersion of the EDA condensate assay for A and NA has been described by Vi tora, Baukal & Wolff (1968)

D 3.3 differential assays: The wavelengths maxima for NA and A fluorophores ar suffi iently apart to permit differential assay by optical means provided no other fluorophores of catechol are present (W il Malherbe & Bone 1953; Persky & Roston 1953) If off-peak wavelengths are used e g 420/510 nm (compared with NA-max of 420/485 nm) to give a NA A fluorescence rati of 2 1; and then e g 420/580 nm (A-max i 420/530 nm) when the ratio is eversed i favour of A (Weil Malherbe 1961) Unfortunately loss of accuracy and sensiti ity with the fluorescenc of A being decreased to ca 10% of that achieved t optimal wav lengths (Udenfriend 1962) makes the assay less suitabl where amounts of the CAs are widely disc epant Other types of single assay procedures tilizing additional differences hav been used One method is based on the photosensiti ity of the NA fluorophore: readings being taken before and 30 mi after exposure of the fluorophores to

light (Keenan Kl ltsch & Humoller 1959) A second method is based on the slower development of the NA fluorophore at room temperature when using CA molybdate complexes eadings being taken 30 ml after condensation t room temperature and agai 30 ml later after heating at 50°C (Nadeau & Sobolewski 1959)

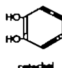
In two-component assay procedure one portion of the eluate was used for assaying total A plus NA and to another portion was added thiosulphate to greatly i hibit (by ca 80%) the formation of the NA fluorophor (see Manger t al 1959) Although division of the eluat immediately halves sensiti ity t least wavelength combinations can be used t which near maximal fluorescenc i obtained.

D 3 4 blanks: ISt and TB are not routinely used i these assays A "PS" i generally used instead of a TB to correct the S eading The final sol ent extraction of the CA fluorophor dimi ishes quenching or potentiation of specifi fluorescenc (see W il Malherbe 1971) and has lso led to an almost total bsence of non-specifi fluorescence from material deri ed from purification procedures ("PS = RB) (see W il Malherbe 1961) and tissues the l tter shown by complexing the CA with borate before processing the tissue (Kagi t al 1957)

D 3 5 sensitivity: Fluorophores formed from CAs i EDA-condensate assays are strongly fluorescent Furthermore the total eluate which contains most of the CA originally present i th biological material may be used i the sampl tube for conversion to the fluorophore no division of the eluat generally being used for ISt or TB The fluorophor i then concentrated i to small volume of organi sol ent Thus the overall assay can be ery sensitive although the lack of accurate blanks may hav disadvantages.

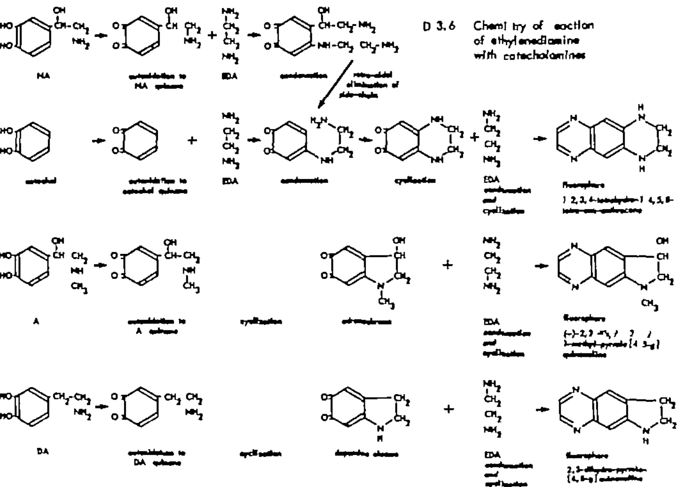
In the 1950 the sensitivity of the EDA-condensate assays was greater than the exi ting HI assays and was consequently often preferred for measuring small amounts of A and NA such as wer i blood plasma However when the assay has to be applied differentially for A and NA considerabl loss of sensitivity may be incurred (see bov) Amounts of A and NA of the order of 10^{-9} g per cuvette ml (e g 1 8 ml) (Vi lora t l 1968; Weil Malherbe 1971) may be determined with similar order of sensiti ity for amines i biological material

D 3.6 chemistry: The chemical basi of the EDA-condensate assay for catechol compounds i the oxidation of the catechol often by disol ed oxygen to quinone derivative which i alkaline solution readily condenses with EDA to form stabl polycycli fluorophore The mechanism of condensation varies for different catechols and one molecul of the quinone derivative may condense with either one or two molecules of EDA depending on the nature of any side-chain. these differences ar shown i the



catechol

formulated sequence of reactions which have been established for the formation of the fluorophores of NA (and catechol itself) and of A: the probabl reaction for DA is also given (see Harley-Mason & Laird



D 3.6 Chemi try of eaction of ethylenediamine with catecholamines

1959)

Provided assay conditions are carefully controlled especially the CA EDA tio one A product and two NA products may be formed (Burn & Fl ld 1956; Harley-Mason & Laird 1959; Weil Malherbe 1960); although large number of compounds can undesirably result from condensation with EDA using different conditions (see Weil Malherbe 1959).

For NA one molecul of EDA condenses with NA quinone in the ing-6 position Cyclization then proceeds using the EDA side-chai after elimination of the NA side-chai by a retro--aldol reaction to form the same derivativ as from catechol and condensation with a second molecul of EDA which occurs using the quinoid groups therefore forms identical fluorophores from NA and catechol (Harley-Mason & Laird 1959- Nagatsu & Yagi 1962) During the condensation reaction of catechol derivatives with EDA elimination of the alkyl side-chain is f cilitated by the presence of a hydroxy group on the carbon atom adjacent to the benzene ring (but elimination does not always then occur e g for A see below) (Harley-Mason & Laird 1959) Thus a number of catechol derivatives may lose thei alkyl side-chai during the EDA-condensate assay to form the fluorophore deri ed from catechol and are thus indistinguishabl from NA i the assay including g α-methyl NA (Carbasil) and d'hydroxymandeli acid (DOMA) (Harley-Mason & Laird 1959)

For A the ide-chai of A quinone cycli es ery rapidly to form adrenochrome and thus it is not cast off i spite of having hydroxy group on the appropri te carbon atom This leaves only the quinoid oxygen groups free for ondensation and only one molecul of EDA is therefor used to form the fluorophor Other N-alkyl ted aminoethanol derivatives lso behave like A i the assay e.g isopropyl NA (Harley-Mason & Laird 1959).

DA and other catechol derivatives with an alkyl ide--chai lacking a hydroxy group on the appropriate carbon atom generally retai thei ide-chai during the condensation reaction (see Harley-Mason & Laird 1959) At least the thylamine derivatives may undergo eaction similar to that of A including DA DOPA and DOPAC and differences i retained side-chains may sometimes be reflected i different wavelength maxima for thei fluorophores Th fluorophores of A and DA have similar wavelength maxima which differ from those of NA (equivalent to the catechol fluorophore) (Laverty & Sharman 1965)

A poi t of interest i that when the CAs were acetylated to thei triacetyl derivatives prior to the EDA-condensate assay the wavelength maxima for the A product became the same as for the NA product being different from that of DA (Laverty & Sharman 1965). During the incubation hydrolysi occurs to form the N-acetyl derivatives and it is possibl that N-acetyl A-quinone may cyclize mor slowly than the corresponding A-quinone and thus allow ondensation with EDA to occur at the ing-6 position followed by elimination of the side-chai to form the fluorophore of catechol N-acetyl-DA-quinone lacks

the hydroxyl group on the appropriate carbon atom and thus presumably retains its side-chain.

D 3.7 specificity The EDA-condensate assay is broadly specific for catechol compounds. When using the assay procedure for A and NA greater specificity is obtained by finally extracting their fluorophores into isobutanol. If both A and NA are present i the same eluate the differences in their behaviour in the assay may be sufficient for their differential but not separate assay (e.g. see section D 3.5). The fluorophores of other CAs will also be extracted the most important interference generally being that from DA; others include isopropyl NA (isoprenaline) N-methyl-DA (epinine) and o-methyl-DA (carbocil) (Kägi et al 1957 Weil-Malherbe 1959 Laverty & Sharman 1965). These are all CAs in which the side-chain is retained but with structural differences which in most cases are insufficient to alter the wavelength maxima of their fluorophores usefully from A (Laverty & Sharman 1965). Other interfering catechol compounds are those which lose their side-chain to form the fluorophores of catchol and are per se indistinguishable from NA; these include not only amines e.g. o-methyl-NA but also non-bases e.g. DOMA and catechol itself (Harley-Mason & Laird 1959 Weil-Malherbe 1959). The isobutanol extraction largely excludes however the fluorophores of those non-basic catechols i which the alkyl side-chain is retained e.g. DOPA and DOPAC (although interference by DOPAC has been reported e.g. Valk & Price 1956; see also Euler 1961). The EDA-condensate assay has been used for other catechol compounds e.g. the catechol acids DOMA and DOPAC may be differentially assayed by recording their fluorescence in both the aqueous and organic phases (Weil Malherbe 1959)

Suitable purification of A and NA (and DA) is most important for increasing the overall specificity In the 1950 the EDA-condensate assay was most widely used following alumina purification In some ways an unfortunate combination since both purification and assay procedures are largely dependent on the catechol nucleus for specificity; although later a weakly acidic cation exchange procedure was incorporated for removing non-bases (see Weil Malherbe 1961. The application of the assay to A and [illegible] plasma has been suggested as recently as 1971 (Weil M[illegible] [illegible]) where its sensitivity is satisfactory and where interference [illegible] normally a problem but even here its specificity may not b[illegible] section D 3.7). DA is rarely separated apart from w[illegible] procedures and when all three CAs are present i[illegible] e.g. THI assay for measuring A and NA has generally [illegible] Weil Malherbe & Bone 1957). Thus [illegible]

applied to [illegible] A and NA in [illegible]
1957) and ev[illegible] [illegible]ue 1956) It
and other ma[illegible] [illegible]ng DA unless a
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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 452

Current Research on the Histochemistry and Function of Biogenic Amines

A Tribute to Bengt Falck

Edited by

CHRISTER OWMAN and ANDERS BJÖRKLUND

LUND 1977

the NA and A fluorophores which in this assay hav the same wavelength maxima this providing a highly sensitiv and specifi method for measuring NA in the CNS (Laverty & Sharman 1965)

D 2 8 Comparison of EDA and THI assays for A and NA: In the early 1950 s both EDA and THI assays were avail ble. However the THI assays were considerably more complex and with additional problems of stability for both Sts and TBs In contrast the EDA assay was simpl and with no blanks and therefore no di lsion of the eluate was required Fluorescence was mor stabl and the assay was considerably more sensitive Its mai ecognised disadvantage was the large i terference by DA. At the time i terest was focused on the low levels of A and NA present i blood plasma, with small blood samples and no measurabl DA present Thus sensiti ity was the important consideration and the EDA assay was consequently often preferred

Published values obtained for CAs i plasma using both types of assay wer rather scattered but as the THI assays were improved values obtained by THI and bioassay tended to be i better agreement whereas those obtained with the EDA assays wer often higher Thus the specificity of the EDA assay was questioned and especially as it was usually applied to eluates from lumina purification which was lso largely specifi for catechol the presence of other i terfer ing catechol was suspected (g Hol bauer & Vogt 1954 Euler 1961) The THI assays wer much more spcific and thei protagoni ts suggested that the lower values obtained were correct (g Valk & P I 1956) However although checks on the specifi ity of analyses using the EDA assays were made i terference by other catechol compounds from blood plasma was rarely erified (see g W ll Malherbe 1959; Manger et al 1959) and when comparisons with the THI assays wer made discrepancies wer often not great (e g. W ll-Malherbe & Bone 1957b) Nevertheless although the EDA assays emained essentially unchanged improvements were l ter made i the purification procedures used e g lumina purification routinely being followed by cation-exchange procedur and greater attention was paid to purity of reagents. This resulted i the values obtained with the EDA assay becoming lower and lower (see Weil Malherbe 1961) Even so the values cited by Udenfriend (1962) were till higher than those obtained with THI assays

Thus doubts concerning th specificity of the EDA assay were never dispelled and meanwhil the problems associated with the THI assays become less and sensitivity was increasing With the advent i 1955 of the spectrophotofluorimeter Interest focused more on NA and 5-HT i the CNS. With the realization of the importance of DA present i the mammalian brain (see Carlsson 1959a) the value of the superior specifi ity of the THI assay for NA was then unquestioned Thereafter HI assays for CAs became the methods of choice for the vast majority of occasions (see e.g Weil Malherbe 1971) and THI assays have been developed with sensitivity exceeding that required for measuring the ery low level of A as well as NA i normal blood plasma (e.g. Campuzano Wilkerson & Horvath 1975).

D 4 TRIHYDROXYINDOLE ASSAY for A and NA:

D 4 1 I troduction For reviews of these assays see g Persky (1955) von Euler (1956 1961), Udenfriend (1962 1969), Callingham & Cass (1963) Callingham (1967), Weil Malherbe (1968 1971) Sharman (1971) and Anton & Sayre (1972)

The classical hydroxyindole (HI) assay is based on the controlled oxidati e cyclization of a catecholamine to its chrome derivative which undergoes an i tra-molecular rearrangement i a trongly alkali sol tion i the presence of a suitabl reducing and tabilizing agent to form stabl fluorescent hydroxyindol The assay was developed by Ehrlén (1948) and Lund (1949) for measuring A added to (procaine) solutions and i blood plasma respectively Shortly thereafter it was lso applied to NA i blood i the first differential trihydroxyindol (THI) assay: 2 component assay based on differences i oxidation of A and NA at different pHs (Lund 1950)

Ehrlén (1948) and Lund (1949) realized from their own and earlier tudies that the transient fluorescence exhibited by A i an alkali e solution (Loewi 1918; Paget 1930; Gaddum & Schild 1934) related to an uncontrolled oxidation by dissol ed oxygen with both formation and degradation of fluorophore molecules occurring simultaneously: th latter eaction was slightly slower such that an accumulation of fluorophore was obtained which however even t its transient maximum reflected only about on quarter of the original amount of A (Lund 1949) This was the basis of early semi-quantitative assays. Thus:

A —oxidized→ A-quinone —cyclized→ Adrenochrome —tautomerized→ Adrenolutine —oxidized→ non-fluorescent degradation products
Adrenolutine = unstable fluorescent THI

dissolved oxygen i alkaline solution

As result of these tudies Ehrlén (1948) and Lund (1949) were able to control this series of reactions by dividing the assay i to separate teps such that a tabl fluorophore was formed lz:

A —oxidized→ A-quinone —cyclized→ Adrenochrome —tautomerized→ Adrenolutine stabl fluorescent THI

oxidizing agent in weakly acid solution + reducing and stabilizing agent in alkaline solution

When A i i a weakly acid solution suitable oxidizing agent can be used for quantitative conversion to adrenochrome which i acid conditions i mor tabl and esi ts further oxidation. Then when making the solution alkaline to allow the almost

Instantaneous tautomerization to adrenolutine Incorporation of a suitabl educing and stabilizing agent prevents further oxidation by excess oxidant and by dissol ed oxygen and so tabilizes the THI fluorophore obtained: an equivalent eaction is undergon by NA (Ehrlén 1948; Lund 1949 1950)

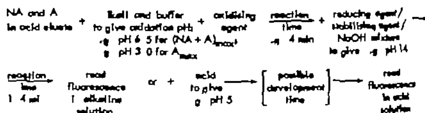
The mai difference between the methods of Ehrlén and Lund was thei choice of oxidising agent: they used ferricyanide and manganese dioxide espectively In both assays ascorbi acid was used as reducing and stabilizing agent The scheme gi en below represents a typical basi for THI assays for A nd NA. I the assays both A and NA can form fluorophores with similar characteri tics and since with most procedures they are purified i to the same eluate differential assays are often required for discriminating between them High discrimination can be achieved by using a 2 component assay procedur with oxidation at two different pHs This is based on the fact that both A and NA can be oxidised over fairly wide pH range but the rate and extent of oxidation of each amine differs with the pH used Both A and NA can be fully and apidly oxidised to thei chrome deri atives t pHs of the order of 5 7 whereas t pHs below 5 the rate of NA oxidation may becom markedly slower than for A. Thus near-maximal A fluorescence may be obtained with low amount of NA fluorescence by oxidising t fixed pH i the egion of 2 4 for fi ed time

A and NA were differenti lly assayed colourimetrically by Euler & Hamberg (1949) using iodine oxidation t pH 6 for formation of both A- and NA-chromes and t pH 4 for complete formation of A-chrome with only littl NA-chrome formation. Lund (1950) adapted thi principl when describing the first fluorimetri differenti l assay for A and NA which used manganese dioxide t two pHs Ferricyanide as oxidant was preferred by Euler & Floding (1955 b and iodine as oxidant i fluorim tri assay was used by Udenfriend & Wyngaarden (1956) where ascorbi acid i lkali was agai used for converting the chromes to tabl i tines.

A imple differential assay procedur but often with lower powers of discrimination was based on differences i the wavelength maxima of each amine Such singl assay procedures wer i troduced by Pri & Pri (1957 ; see lso Carlsson Rosengren Bertler & Nilsson 1957) usi g ferricyanide as oxidant and by Cohen & Goldenburg (1957) using manganese dioxid as oxidant Subsequently ferricyanide and iodine hav become the most widely used oxidants i THI assays for A and NA

D 4.2 optima general There are ery large number of modified ersions of THI assays which hav been dev loped i efforts to increase sensitivity to increase the curacy of differential assays with the ultimate im of producing separate assays for A and NA and to overcome the many local vari tions effecting the complex chemi try f the reaction including the adaptation of an assay to different purification procedure (see lso Weil Malherbe 1968) Before discussing specifi detail of some of these variations some general features of THI assays

will be mentioned; some of the optimal conditions for A and NA are different.



CAs are usually purified i to acid eluates for increased tability A portion of the luate is adjusted to a fixed pH below neutral for oxidation This provides more stabl conditions both for the CAs before idation and for their chrome derivatives. A fixed pH in the range 5 7 ("high pH) i used to obtain near-maximal fluorescence from NA and often also from A; and a fi ed pH i the range 2 4 ("low pH) may lso be used to obtain equally trong A fluorescence but with considerably weaker NA fluorescence For the pH adjustment of the eluate the lkali often required should be added carefully to decrease the risks of spontaneous oxidation due to the creation of local areas of high pH i the solution; K_2CO_3 $NaHCO_3$ Na_2PO_4 or NH_4OH usually being preferred to NaOH or KOH (e g Bertler et al 1958; Euler & Lishjako 1959) Preferably the CAs are not kept at this pH for longer than about 10 minutes and this tends to limit the number of eluates which can be assayed in parallel (Vendsalu 1960) Throughout the purification and assay procedures the isks of spontaneous oxidation of the CAs ar decreased by thei mi imal exposure to near neutral to lkaline pHs and by avoiding high temperatures and sunlight Portions of thi pH-adjusted eluat are taken for S and ISt (and sometimes for blanks see below) and a suitabl buffer is usually added to reinfor the requi ed pH The optimal concentration of oxidising agent is then added The time llowed for oxidation fixed between 1 and 5 minutes depends on the pH and which oxidi ing agent i used and for which CA. The relati rates of A and NA oxidation may be modified intentionally by the use of metal ions as a catalyst; but also sometimes undesirably by agents derived from tissues and purification procedures.

The choic of educing and stabili ing agent for which one or two compounds may be used depends upon the oxidant the final pH for fluorescence and the CA. Sometimes an agent is used which stabilizes the fluorophor of NA mor effecti ely than that of A for differential assay purposes. NaOH i usually used to give the optimal pH of greater than 13; the amount required depending on the buffering capacity of the pH adjusted eluate Optimal amounts of reducing and tabili ing agents and NaOH are usually fully effectiv if added as a mi ture although sometimes the reducing agent is added first The eaction i then llowed to proceed for a fi ed time usually between 1 and 5 minutes

Fluorescence i then re orded: ither i the alkaline sol tion or in a sol tion acidified usually with c tic acid to an

optimal fixed pH I the range 4 5 in which case there is a shift to shorter wavelengths This wav length shift has important implications for specificity (see section D 4 5) Depending on the oxidant used and the CA maximum fluores enc is immedi tely available for measurement or may requi e development time This time may be shortened by i troducing energy such as heat or light, once fluorescence has fully developed it is usually ead withi fixed time i terval depending on the tability achieved

For early THI procedures great dil tion of the eluate was requi ed to decrease the effect of quen hing by material from purification procedures especially lumina also availabl filter-fluorimeters used large cuv tte volumes Thus the proportion of eluat to assay reagents was often small (g 0 5 ml portions of eluate in final cuv tte olumes of 10 ml Euler & Floding 1955b) However material used i purification procedures are now generally i a higher tate of purity including alumina whi h in addition i more frequently followed by a secondary purification procedur for the CAs Also improvements in instrumentation have enabled precise wavelengths and smaller cuv tt volumes to be used Thus i recently modified form of the abov assay 0 85 ml portions of eluate wer used i final cuvette olumes f 1 55 ml (Keh Carlsson & Lindqvist 1976)

T simplify consideration of some of the numerous versions of the THI assays availabl for A and NA they will be di ided i to four groups according t the types of oxidant and reducing/ /stabilizing agents used

D 4 2 IODINE as oxidant plus THIOSULPHATE / ASCORBIC ACID as reducing/ tabilizing agents

Thi type of assay first used by Udenfriend & Wyngaarden (1956) (also by Sourkes & Drujan 1957; and Shore & Olin 1958) was presented i mor d tail by Crout (1961) In thi group f assays oxidation may be performed with iodine t fixed pH i the region of 6 for A nd NA; also t fi ed low pH of bout 3 particularly for A Sodium thiosulphat was used to reduc excess iodine and ascorbi acid to tabili the fluorophores which it does mor effi iently i lkaline sol tion Fluorescence which develops slowly was therefor generally recorded i alkaline solution If iodine i preferred as oxidant then this group of assays generally lacks the advantages of the following group of assays developed l ter (section D 4 2b) whi h are simpler to use and do not have the problems associated with the use of ascorbi acid (section D 4 2) (see g Laverty & Taylor 1968; also section D 4 2b)

NA and A eluate pH adjusted and buffered	iodine oxidant	wait e.g. →	$Na_2S_2O_3$ reducing agent	wait e.g. →	ascorbic acid stabilizing agent / NaOH / alkali mixture	energy e.g. UV light for →	read
e.g. pH 6		5 min		5 min		10 min	NA_{max} 395/505 nm
e.g. pH 3.5		5 min		5 min		10 min	A_{max} 410/

Oxidation at High pH for NA and A: In assays using oxidation at pHs of this order high fluorescence intensities from both A and NA be obtained (between 0 5 : 1 and 1 1) The eluate was usually adjusted to a fixed pH buffered with acetate buffer (g Welch & Welch 1969) I the range ↄ 7 for oxidation EDTA has been added to urine alumina eluates to prevent a precipitate from causing severe quenching I the assay (Crout 1961); lthough the presence of EDTA during the oxidation has been reported to decrease fluorescence and induce higher blanks (Welch & Welch 1969) Iodine was then added and oxidation occurred rapidly but fixed time of 5 minutes was generally llowed before adding the thiosulphate reducing agent Then the ascorbi acid and NaOH were added as a freshly-prepared mi ture To decrease non-specific fluorescenc contributed by ascorbic acid the use of 10 N NaOH (instead of 5 N NaOH) and of thylene diamin (see se tion D 4 2) hav been adopted (Welch & Welch 1969) Fluorescence developed ery slowly for both A and NA products reaching a maximum I about 45 minutes in daylight The quality of the daylight could effect thi development (Crout 1961; Chi Picchioni & Childs 1963) and irradi tion using standardized light conditions gave mor intense and reproducible fluorescence (Sloan Brooks, Eisenman & Martin 1962) g fter 5 minutes irradi tion with UV light fluorescenc was increased by ca 25% for NA and ca 8% for A (Kahane & Vestergaard 1965) However fluorescence was then only table for less than 10 minutes as compared with more than 45 minutes using weaker light sources (Crout 1961; W I h & Welch 1969) Possibly a slightly weaker I radiation may provide a rapid inc ease I fluorescence together with greater stability; lso the A fluorophore may require less irradiation than the NA fluorophor (see section D 4 2b)

Oxidation t Low pH for A (plus NA) I ssays using oxidation at a fi ed pH of this order high fluorescenc intensity from A was maintained but with a I w NA I terferen e Thi was often of the order of 7% after oxidation I sodium acetate buffer t pH 3 5 but if a sodium phosphate/metaphosphate buffer t pH 2 85 was used I terference may be decreased to ca 0 7% possibly due to the presence of metaphosphori acid or an impurity I the buffer which may selectively suppress the formation of the NA I tine (Kahane & Vestergaard 1965) The other features of these assays were essentially as described for the high pH oxidation (e g Crout 1961)

Differential Assay for A and NA Discrimination between A and NA has gener lly been made using oxidation at two pHs in a 2-component assay coupled with differences I wav length maxima The index of discrimination for A NA obtained by Kahan & Vestergaard (1965) was as high as 300 (A-max/NA-max 150/0 5)

An automated ersion of the Low pH assay for A based on the pH 2.85 m taphosphate buffer has been described (Honig Morrison & Krop 1972)

D 4.2b IODINE as oxidant plus SULPHITE/EDTA as reducing/ tabili ing agents

This type of assay was introduced by Chang (1964) for A and NA as well as DA following the earlier development of a similar assay by Carlsson & Waldeck (1958) for DA alone (see section E 4) The arious steps i the assay for these three amines and also several el ted compounds were studied i d tail by Laverty & Taylor (1968) who showed how the optimal conditions varied for each compound These differences wer further studied and utili ed i sensitive assays for A and NA i blood plasma by O Hanlon Campuzano & Horvath (1970) Griffiths Leung & McDonald (1970) and Campuzano Wilkerson & Horv th (1975)

In thi type of assay oxidation with iodine may be per formed at fi ed pH close to 6 5 for A and NA; lso sometimes t a lower fi ed pH around 4 particularly for A. Sodium sulphite and EDTA ar used as reducing and stabili ing agents; and final acidification to ca. pH 5 i lways mad for increased i tensity and tability of fluorescence; this combination of factors lso results i much lower and more tabl blank fluorescence as compared with that generally obtained when using ascorbi acid after iodine oxidation (see D 4 2 above) Fluorescence of A is maximal almost immedi tely whereas development tim i requi ed for maximal fluorescence from NA (and also from DA nd most other rel ted compounds) These assays ar simpl straightforward and generally reliabl nd some of the most sensitive ersions of the THI assays for A and NA are to be found i this group

NA and A eluate pH adjusted and buffered	iodine oxidant (wait)	Na_2SO_3/ EDTA /NaOH reducing/stabilising/alkali agent solution (wait)	acetic acid (to)	development time	read
				20°C for	
e pH 6 75	3 min	5 min	pH 4 8	25 min	NA_{max} 380/480 nm
e pH 3 8	3 min	1 min	pH 5 0	none	A_{max} 410/500 nm

Oxidation at High pH for NA (plus A): In assays of thi type fluorescenc tends to be higher from NA than from A and they can be optimised for high NA fluorescence with moderately low fluorescence from A (e g 18% A i terference)

The eluate was adjusted to a fixed pH g. 6.75 (O Hanlon t l 1970) phosphate buffer seeming to give the best results (Laverty & Taylor 1968) The optimal amount of iodine was then added and allowed to act for an optimal time g 3 minutes (Laverty & Taylor 1968) before adding as mi ture the optimal amounts of Na_2SO_3, EDTA and NaOH (Campuzano t al 1975) The presenc of EDTA seemed to be essential possibly for tabili tion of the i (Welch & Wel h 1969) The EDTA was often incorporated ea

the assay procedure usually i the buffer especi lly when using lcoholic iodine sol tions for oxidation (Chang 1964, Ansell & Beeson 1968); in this case it appeared to serv the additional function of stabilizating the intermediate products of oxidation (Karasawa Funakoshi Furukawa & Yoshida 1973) After allowing an optimal time g 5 minutes for tautomerization to occur the sol tion was acidified usually with acetic acid to the optimal pH g 4 8 since the lutine was better stabili ed i such conditions (Laverty & T ylor 1968) Acidification esulted i a wavelength shift for NA from 390/505 nm in alkali to 380/480 nm i acid with a concomitant increase i sensiti ity and specificity (Laverty & Taylor 1968; see also section D 4 2) The development time equired for maximal NA fluorescence was bout 25 minutes t room temperature (Laverty & Taylor 1968) and fluorescence was stabl for nother 30 minutes (e g O Hanlon et al 1970) It may be an advantage to use standardi ed conditions of temperature and lighting during this development time

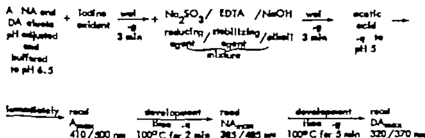
Oxidation at Low pH for A (plus NA) In assays using oxidation t a low pH a high fluorescence intensity may be obtained from A with ery littl i terfe enc from NA (e g 8% Laverty & Taylor 1968 to 0.4% Campuzano et al 1975) The assay i i outline as described abov for NA However citrate or citrate/phosphate buffer has been found to give better results (Laverty & Taylor 1968; O Hanlon t al 1970) and the optimal amounts of Na_2SO_3 EDTA and NaOH may differ (e g Campuzano et l 1975) Also a very much shorter time for tautomerization was requi ed generally 1 minute before final acidification to slightly highe pH of 5 0 (Laverty & Taylor 1968) Fluorescenc developed quickly and was read almost immediately lthough the fluorophore was found to be stable for more than 30 minutes provided optimal conditions for the A assay were carefully adhered to (O Hanlon et l 1970; Campuzano et l 1975).

I thei ery sensiti e assays for A and NA Campuzano and co-workers (1975) presented consider ble detail for establishing thei assays and for preparation of many of thei solutions i bulk for long-term freeze-storage

Differential Assays for A and NA: Very high discrimination between A and NA with high sensiti ity may be obtained by using a 2-component assay based on oxidation at two different pHs Each assay can be used optimally for maximum fluorescence keeping mutual interference to minimum Thus not only i NA i terference low i the Low pH" assay for A but A interferenc i greatly decreased i the High pH assay for NA gi ing an index of discrimination for A NA of 1400 (A-max/NA-ma 250/0 18) (Campuzano et al, 1975) The sensitivity of these assays i generally of the order of 10^{-9} g amine per cuv tte ml However when assay conditions are fully optimi ed very sensitive assays may be evol ed Thus for A sensitivity of the order of 10^{-13} g per cuv tte ml (0 5 ml luat portion i 2.0 ml total volume) was eported. With 1 5 ml eluat 10^{-12} g A i tissue may be measured. For NA equi alent onditions may gi sensiti ity of the order of 10^{-12} g per cuvette ml and 10^{-11} g i tissue These are higher orders

of sensitivity than are required to measur A and NA i blood plasma (see Campuzano et al 1975)

Differential Assay for (A) + NA + DA Differences i development and fading tim i addition to the use of wavelength maxima for each amine provide an i teresting approach for discrimination between A NA and also DA. The f t that these CAs ar often purified into the same eluate is thus used to advantage sinc i a singl assay procedure three separate fluorescenc recordings may be made for the three amines (Chang 1964) However the procedure has not been widely used for measuring A.



The fairly long development time for NA and even longer tim for DA are often shortened by using a mor i tense source of energy Thus A fluorescence may be read immediately t its wavelength maxima the assay sol tion may then be heated i water bath at 100°C for 2 minutes before recording NA fluorescence and finally the assay solution may be heated for a further 5 minutes before reading DA fluorescence at its wav length maxima (Chang 1964) However discrimination between A and NA i not so good because NA fluorescence will be present and increasing whil t eading t the A wavelength–max. (410/500 nm) and A fluorescenc will still be present whilst reading at NA wavelength–max (385/485 nm) (see Chang 1964) Thi sam effect mai ly between NA and DA is of less consequenc because DA wavelength–max. (320/370 nm) re considerably shorter than the NA wavelength–max t the acid pH The specifi energy requirements may vary between laboratories and should preferably be checked to ensur maximal fluorescence of each amine with minimal i terference from the ther two amines. A small gai i sensiti ity may possibly be obtained relati ely easily by adjusting the final pH to g pH 4 8 before developing and reading NA fluorescence and then further adjusting t g pH 4 4 before eading the DA fluor escence (*these being the optimal pHs determined by Laverty & Taylor 1968*) Neverthele loss of sensiti ity i incurred because of the compromise i assay conditions prior to the final acidification i particular the time allowed for tautomerization i 2 minutes instead of 1 minute for A or 5 minutes for NA and DA: lso the amounts of NA_2SO_3, EDTA and NaOH which optimally differ for each amine (Laverty & Taylor 1968; Campuzano t al 1975) Poor stabilization of A fluorescenc has been reported (Welch & Welch 1969); optimal conditions wer used (Campuzano t al 1975) Thus this

approach for differentl lly assaying the three amines I torduced by Chang (1964) has I practic been most commonly incorporated I to procedures for analyzl g NA and DA (e g Ansell & Beeson 1968· Shellenburger & Gordon 1971)

D 4 2c FERRICYANIDE as oxidant plus ASCORBIC ACID as reducing/ / stabilizing agents

Thi type of assay was introduced by Ehrlén (1948) and was modified and pplied to tissues by Euler & Floding (1955) (for d tailed descriptions of assays see I addition e g Pri & Pri 1957; Bertler t 1 1958; schaepdryver 1958 Euler & Lishjako 1961; Anton & Sayr 1962; Peyrin & Cottet-Émard 1973)

In this group of assays oxidation may be performed with ferricyanid t a fi ed pH in the region of 6 for A nd NA: also sometimes t a low fi ed pH of bout 3 particularly for A Ascorbic acid i used as educing agent and stabili es th fluorophores more effi iently i lkaline solution Fluorescenc which develops rapidly is therefore generally recorded i the alkali e solution The assay tends to be simple straightforward and fairly r liable except when measuring fluorescence t levels approaching bl nk values when the non-specific fluorescence contributed by ascorbic acid becomes of greater consequence

NA and A eluate pH adjusted and buffered	[zinc-sulphate] +	ferri-cyanide oxidant	wait →	+ ascorbic acid / NaOH reducing-stabili ing/alkali agent mixture	→ read
-g pH 6 5			3 min		NA_{max} 410/535 nm
-g pH 3 0			3 m[illegible]		A_{max} 425/545 nm

Oxidation t High pH for NA and A: In assays using oxidation t pHs of this order high fluorescen I tensities from both A and NA can be obtained usually of the order of 1 7 1 to 1 1 i e fluorescence tends to be slightly greater for A than NA (although Anton & Sayre (1962) eported a ratio of 0 6 1)

The eluate i usually adjusted to fi ed pH phosphate buffer seeming to gi e the best esults (Anton & Sayre 1962) i the range 5 5 — 7 0 for oxidation The optimal amount of ferri yanide i then added (Euler & Floding 1955) The presence of zinc sulphate has been eported to gi more eproducibl esults (Bertler t al 1958) Oxidation has been shown to occu rapidly e g i less than 2 minutes at pH 6; but fi ed time usually between 2 and 5 minutes is generally allowed before adding the alkali (Ehrlén 1948; Euler & Floding 1955a) although a time i terval as short as 1 minute has been preferred and only one eluate at a time was assayed (Anton &

Sayre 1962) The optimal amounts of ascorbic acid and N OH are added most commonly as freshly-prepared mi tur (e g Ehrlén 1948; Euler & Floding 1955a) this may giv increased fluorescence i tensity (see Weil Malherbe 1968) as compared with adding them consecutiv ly (e g Pri e & Pric 1957) *Th time required for maximal fluorescenc* to develop (1 – 10 minutes) and th most suitable time for ecording *fluorescence (1 15 minutes) i alkaline solution hav* varied (see g. Ehrlén 1948; E ler & Floding 1955a- Vendsalu 1960; Anton & Sayre 1962) here the changing i tensity of the blank fluorescence should lso be considered (e g Vendsalu 1960) The addition of aceti acid to give pH 5 has occasionally been used t increase fluorescence intensity *of the A and NA fluorophores* (and lso to decrease the i terference of some other rel ted compounds) but t the expense *of less tabl fluorescenc whi h equi es mor care i timing* of readings (P ic & P ice 1957)

Automated ersions of the high pH assay for A and NA hav been described (Merrill 1963; Robinson & Watts 1965; Martti & Harrison 1968)

Oxidation at Low pH for A (plus NA): i assays with oxidation t the *lower pHs* of the order of 3 high fluores en *is obtained from A* but with low fluorescenc from NA (generally 3 10%)

Oxidation by ferri yanide t lower pHs g. i the range 3 4 i much lower for NA *than A and has proved useful* when wishing to minimi i terfering fluorescence from NA (Euler & Floding 1955a) Aceti acid has been preferred to the commonly used acetate buffer to gi the low pH (Anton & Sayre 1962); also the metaphosphat buffer (see section D 4 2) has been adopted (Peyri & Cottet-Emard 1973) Zink sulphate has sometimes proved useful to talyz the reaction g for oxidation t pH 3.5 addition of optimal amounts of ferri yanide and inc sulphate with ea tion for 3 minutes resulted i the same amount of A fluorescenc as at pH 6 *but less than 5%* NA fluorescence (Euler & Floding 1955) However catalysts hav not lways been found ecessary even when using *oxidation t pHs as low as* 2 0 2.5 (g Anton & Sayre 1962, Peyri & Cottet-Emard 1973) There seems to be considerabl variation *between i boratories as to the need for* catalyst and *the most suitabl* onditions of pH and buffer requi ed to chiev high fluorescence from A with litt *NA interferenc Moreover t whatever fixed low pH* is used with or without catalyst other agents derived from tissue and purifi ation procedures *may lso modify the el tiv rates of A* and NA oxidation such that although the ratio of NA A fluorescence for th St* i very *low for the S It may be unacceptably higher* (e.g W ight 1958) T check whether thi problem exists on IStˢ *not only* of A but also of NA *can be compared with appropriate* external Stˢ t least i early experiments The ther features of the assay are *essentially as described for the "High pH"* assay (g see Euler & Floding 1955a; Peyri & C ttet-Emard 1973)

An automated version of the "Low pH" assay for A utili ing the pH 2.8 metaphosphate buffer of Kahane & Vestergaard

approach for differentially assaying the three amines I tordu ced by Chang (1964) has I practice been most commonly incorporated into procedures for analyzing NA nd DA (e g Ansell & Beeson 1968; Shellenburger & Gordon 1971)

D 4 2c FERRICYANIDE as oxida t plus ASCORBIC ACID as reducing/ /stabilizing agents

This type of assay was introduced by Ehrlén (1948) and was modified and applied to tissues by Euler & Floding (1955a) (for detailed descriptions of assays see i addition e g P ice & Price 1957; Bertler t al 1958; Schoepdryver 1958; Euler & Lishajko 1961; Anton & Sayre 1962 Peyrin & Cottet-Émard 1973)

I this group of assays oxidation may be performed with ferricyanid t fixed pH I the region of 6 for A nd NA also sometimes t a low fi ed pH of about 3 particularly for A Ascorbic acid is used as educing agent and tabili es th fluorophores more effi iently in lkaline solution Fluorescenc which develops rapidly is therefore generally ecorded in the lkaline solution The assay tends to be impl traightforward and fairly reliable except when measuring fluorescence at levels approaching blank values when the non-specifi fluorescence contributed by ascorbi acid becomes of g eater consequence

NA and A luate pH adjusted and buffered + [zinc- sulphate] ferri- cyanide oxidant —wait→ + ascorbic acid / NaOH ——→ read
reducing- stabilising / alkali agent mixture

pH 6 5	3 ml		NA_{max} 410/535 nm
pH 3 0	3 ml		A_{max} 425/545 nm

Oxidation t High pH for NA and A: I assays using oxidation at pHs of this order high fluorescenc i tensities from both A and NA can be obtained usually of the order of 1 7 1 to 1 1 i e fluor- escenc tends to be slightly greater for A than NA (although Anton & Sayr (1962) eported a ratio of 0 6 1)

The eluate is usually adjusted to a fi ed pH phosphate buff seeming to giv the best esults (Anton & Sayre 1962) in the range 5 5 — 7 0 for oxidation The optimal amount of ferricyanide is then added (Euler & Floding 1955a) The presenc of inc sulphate has been eported to gi more reproducibl results (Bertler t al 1958). Oxidation has been shown to occur rapidly g i less than 2 minutes at pH 6; but fixed time usually between 2 and 5 minutes is generally allowed before adding the alk li (Ehrlén 1948; Euler & Floding 1955) although time interval as short as 1 minute has been preferred and only one eluat t a time was assayed (Anton &

As S approaches TB calcul tions of net fluorescence ("S TB) therefore can become increasingly inaccurate T minimise these problems various steps have been taken

1) The ascorbic cid is freshly prepared (e g Anton & Sayre 1962); and the ascorbl acid NaOH mi ture i prepared immediately before use i e after adding the oxidi ing agent during the assay procedure (Euler & Floding 1955a)

2) Ascorbl acid is dissol ed i 10 N NaOH (g 0 2 pa ts of 10% ascorbl acid mi ed with 10 parts 10 N NaOH Anton & Sayre 1962)- instead of i the more usual 5 N NaOH (e g 1 part 2% ascorbl acid mi ed with 9 parts 5 N NaOH e g Euler & Floding 1955); the improvement may be rel ted to an increased tate of ionization i 10 N NaOH of the two hydroxy groups of the enediol form of ascorbl acid which may give greater reducing capacity t the instant t which luti formation occurs (Weil Malherbe 1968)

3) Various agents hav been used to stabili ascorbl cid i alkaline solution e g thylene diamine (Euler & Lishajko 1961; W il Malherbe & Bigelow 1968); β-thiopropionl acid (P iner 1963); sodium borohydride (Gerst Steinsland & Walcott 1966); and 3-mercaptopropioni acid plus Na_2SO_3 (Marti & Harrison 1968)

4) Afte adding the ascorbl acid / NaOH mixture the assay solution should be mixed immediately and thoroughly (Anton & Sayre 1962) but not too igorously g. by tapping the tube gently (Vendsalu 1960) rather than using ortex mixer This may help to prevent i being dissol ed which accelerates the breakdown of ascorbic acid

However when equi ing to measure very low concentrations of CAs assay procedures avoiding the use of ascorbl id may be preferred (see below D 4. 2d)

D 4. 2d FERRICYANIDE as oxidant plus SULPHYDRYL compound as stabili ing agent:

I this type of assay ferricyanide is agai used as oxidant However sulphur-contai ing compounds have been chosen to replace ascorbl acid as the tabilizing agent but with two different objecti es i mind Haggendal (1963) sought to increase sensiti ity by obtai ing low and tabl bl nk fluorescence (which was high and variabl i ferri yanide / ascorbl acid assays see section D 4 2 bov) and found mercapto-alcohol to be particularly suitable; cysteine has also been used (Klensch 1966) Merrill (1962) sought an agent which would preferentially tabili the NA fluorophor (I contrast to ascorbl acid which equally tabilized both the A and NA fluorophores see section D 4. 2) and found thioglycollic acid to be effective Both these types of assay have been developed and hav proved valuable- although care i generally equired to prevent contamination by the sulphur-containing compound from inhibiting amine fluorescenc

Ferricya ide as oxidant and mercapto-alcohol as stabili ing agent:

Using a mercapto-alcohol i place of ascorbic acid increased overall sensitivity resulted from low and stable blanks even though when reading i alkaline sol tion THI fluorescence was not increased. Also a second activation peak for both A and NA fluorophores was obtained these having been obscured (by UV-absorption) when using ascorbic cid. The presenc of two activation peaks for each fluorophore is very useful for confirmation of identity when measuring small amounts of these amines (Häggendal 1963a).

Using a final acidification fluorescence was considerably i tensified and made more stabl (W il Malherbe & Bigelow 1968; see also Valori Brunori Renzini & Corea 1970) (In contrast to ferricyanide/ /ascorbi acid assays wher acidifi tion gave increased but less stable fluorescence Pri e & Price 1957). The advantages of low and stabl blank nd of two activation wavelength peaks for each i tine were retained. These mercapto-alcohol may not stabilize the A fluorophore so effectively i lkaline solution this iew being supported by the use f ther sulphydryl compounds to tabili only the NA fluorophore in alkaline solution (see below). This may expl in why Häggendal (1963) could not d tect A i normal blood plasma with an assay which is widely regarded as one of the mor sensiti THI assays for NA. Thus in acid sol tion THI fluorescence is increased and blanks are low and more stable gi ing a considerabl overall increase i sensitivity; although the THI fluorescenc has a tendency to slowly decline (compared with ferricyanide / ascorbic acid assays).

NA and A eluate pH adjusted and buffered + Cu^{++} ferricyanide oxidant $\xrightarrow[\text{e.g.}]{\text{wait}}$ + Na_2SO_3/ mercapto-alcohol /NaOH or formaldehyde/ mercapto-alcohol /NaOH mixture $\xrightarrow[\text{e.g.}]{\text{wait}}$ acetic acid e.g. to → read

e.g. pH 6.5 3 min 4 min pH 5.0 NA max 395/475 nm

e.g. pH 2.8 5 min 4 min pH 5.0 A max 415/500 nm

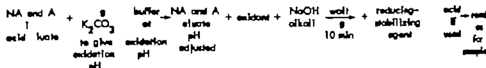
Oxidation at High pH for NA and A: In assays using oxidation at pHs of the order of 6 7 high fluorescence intensity from NA may be obtained with lower fluorescence g. 30% from A (Weil Malherbe & Bigelow 1968). After adjusting the eluate to a fi ed pH the optimal amount of ferricyanide is then added and oxidation occurs rapidly but fi ed time between 3 and 5 minutes i usually allowed. The presence of Cu ions during oxidation seemed to increase reproducibility when using alkaline sulphit as educing agent and mercapto-al ohol as tabili ing agent (Häggendal 1963a; Weil Malherbe & Bigelow 1968). However the presenc of Cu^{++} ions during oxidation gav four fold increase i A fluorescence when using a tronger sol tion of mercapto-alcohol i formaldehyde (the sulphite being omitted)

(Valori et al 1970) Dimercapto-propanol (BAL) (used by Häggendal 1963a; and Valori t al 1970) has a low water solubility and great care is needed to ensure its complete emoval from glassware Mercapto-ethanol may be easier to obtai I more consistantly pure state than BAL (Weil Malherbe & Bigelow 1968) and although it is water solubl It I lso more olatil and thus equires great car to avoid ontamination during an assay The mercapto-alcohol I dissolved I ither the sulphite or formaldehyde solution. Thi I then added either immediately befor (Häggendal 1963a- V lori t l 1970) or more conveniently mi ed with the NaOH (see Weil Malherbe & Bigelow 1968; Häggendal personal communication) The stabili ed fluorophores each showed two different ctivation wavelengths: I alkaline solution these were for NA-max 395/515 nm and 330/515 nm and for A-max 420/525 nm and 330/525 nm (Häggendal 1963)

A final acidification to g pH 5 0 after allowing tautomerization to proceed for fi ed time g 4 minutes resulted I a 50% increase I fluorescence and helped to stabili the NA lutine (W il-Malherbe & Bigelow 1968; Valori t al 1970) The pH adjustment lso resulted I a shift of fluorescence wavelength maxima from 515 to 475 nm for NA and from 525 to 500 nm for A fluorophores; the activation wavelengths remaining essenti lly unchanged (Weil-Malherbe & Bigelow 1968) However the fluorophores were still not well stabili ed fluorescence tending to decli very lowly g by 5% after 30 minutes (Weil Malherbe & Bigelow 1968); although using tronger concentrations of BAL i formaldehyde (omitting the sulphite) fluorescenc was reported to be tabl for two hours (Renzini t l 1970)

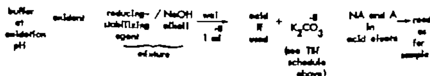
Oxidation t Low pH for A (plus NA) In thi assay using oxidation t pH 2.8 as described by W il Malherbe & Bigelow (1968) high fluorescence I tensity was obtained from A with only 7% I terference from NA.

Formi acid was used to achiev the low pH for oxidation with ferricyanide although other acids e.g. citri and phosphori acids gav similar results. However the use of metaphosphate buffer to inhibit NA oxidation (as eported by Kahane & Vestergaard 1965) lso inhibited oxidation of A I this assay: probably by chel ting the Cu^{++} ions. The pH of 2.8 littl below the optimal pH of 3 for A oxidation was chosen to minimi I terference from NA since the use of Cu ions to obtai ten-fold increase I A fluorescence lso catalyzed NA fluorescence I the region of pH 3 However other material probably derived from purification procedures and tissues also influenced the relativ rates of oxidation of A and NA t pHs near 3 and produced anomolous esults (see W il-Malherbe & Bigelow 1968) After tautomerization final acidification to pH 5 esulted I 50% increase I fluorescence after a white copper precipitate was removed by brief centrifugation. The THI fluorescenc from A declined slowly 12% loss occurring I 30 minutes; readi were made withi 20 minutes f acidification and ISt may be used correct the S However using thi Low pH" assay for A



endogenous reducing agents passing i to the CA eluate and so pre-enting compl te fading of the CA-derived fluorescence: this may be erified by spectral recordings

TBu procedures i which oxidation of the CA should be compl tely prevented l ter became more commonly used than the TBf proced res However parti l f llur frequently occurs resulting i high blanks due to erifiable specific amine-derived fluorescence Several different schedules for TBu have been used. Frequently to the pH adjusted eluate i added; first the reducing/stabilizing agent followed by the oxidising agent (i some assays the oxidant i entirely omitted c.f P ice & Price 1957) and finally the NaOH lone (e g. Laverty & Taylor 1968; Shellenberger & Gordon 1971) The reducing/ /stabilizing agent is added first i an attempt to prevent oxidation of the CAs both by dissol ed oxygen i the N OH and by the oxidant itself (Pri & P i 1957) However spontaneous oxidation of the CAs by dissol ed oxygen i local reas f high pH may often occur during pH adjustment of the luate (e g P i & P ice 1957; Vendsal 1960; Laverty & Taylor 1968) (the risk bei g exacerbated i conditions of high ambient temperatures and strong sunlight) The problem may be decreased by using the acid eluate i partly eversed schedul (e g. Welch & Welch 1969) the amount of alkali e g K_2CO_3, used i the S for adjustment to the oxidation pH being added as the last tep (usually indi idual portions of a sufficiently large volume of pH adjusted eluate are taken for S ISt and TBf if used but the proportions of acid eluate and K_2CO_3 can be calculated and used separately i the TBu procedure)

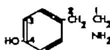


Perhaps the best TBu procedure scheduled above i which the isks of spontaneous oxidation ar decreased is to mix all the assay reagents together i the normal order including the volume of g. K_2CO_3 which was equi ed for adjustment of pH i the S before finally adding the acid eluat containing the CAs (e g Chang 1964,

O Hanlon et al 1970) This type of blank is ery independent of the CA content of the eluate It also has an added advantage of convenience i most HI assays as compared with the TBf and most ther TBu procedures: the same mi tur of reducing/ tabili ing agent i NaOH as is used for St and S can also be used for the TBu

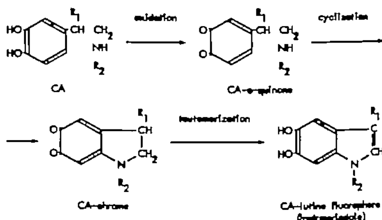
choice of blanks Some material which i non-fluores ent may be oxidized i the HI assay procedure to giv interfering fluorescence whil t other material which gives nati i terfering fluorescenc i the assay may be destroyed by excess oxidation i the TBu S and TBf procedures i the HI assay the oxidation times may be g. 0 5 and 10 minutes respectiv ly: if the abov types of material re present i the eluate then ither too much or too littl i terfering fluorescence may be present i the TBu or TBf as compared with the S (e g see discussion by Häggendal 1963 ; also section E 4 3) The best TB procedure is that i which the TB has value nearest the S* when using different amounts of a tissue which has been deprived of only its CA content (g Chang 1964) and may vary depending on the type of tissue purification and assay procedure used. However the TBu procedur i which the acid eluate is added after pre-mi ing the assay eagents does appear to be generally one of the best TB procedures as well as the most convenient

4 chemi try: The chemical basi of hydroxyindol assays appears to be that compounds having a 2-(4-hydroxyphenyl)-ethylamine nucleus with certai substituents can be converted by oxidati cyclization and autoreduction to fluorescent hydroxyindol (HI) derivati es For fluorophore formation the 4-hydroxy group appears to be essential as is the amino group which may be primary or secondary However substitution i the 3- ing position by hydroxy group results i considerabl increase i fluorescenc i tensity: somewhat smaller increase resulting from 3-methoxy group substitution: thus fluorophores may be formed from catechol (ethyl) amines and derivati es including 3-O-methyl ted derivatives Examples of suitabl substitutions i the ide-chai include: hydroxy group i the 2-position (i the β-position) g as i NA and NMy methoxy or carboxy group or both i the 1-position (i the α-position) g. as in α-methyl-DA DOPA and α-methyl-DOPA; and an α-methyl group i the N-position g as in N-methyl DA (epinine) and A (see Laverty & Taylor 1968; also Anton & Sayre 1964)



2-(4-hydroxyphenyl)-ethylamine

The form l ted sequence of rea tions shows the conversion of the CAs to th l espect HI fluorophores This is a very simplified epresentation of the chemi try of the reaction which has been the focus of considerabl investigation (see g Ehrlén 1948; Lund 1949; Fischer 1949 Harley-Mason 1950; Bu Lock & Harley-Mason 1951; Harrison 1963 Harrison et al 1968; see lso Euler 1959)



for A	R_1	OH	R_2	CH_3	fluorophore	1-methyl 3,5,6-trihydroxyindole
for NA	R_1	OH	R_2	H	fluorophore	3 5,6-trihydroxyindole
for DA	R_1	H	R_2	H	fluorophore	5,6-dihydroxyindole

This type of rea tion occurs when ferricyanide is used as oxida t When using iodine (or periodate) however the iodine itself may be partially incorporated l to the reaction so that 2-iodo-quinones chromes and indoles are also formed (Ehrlén 1948b; Bu Lock & Harley-Mason 1951)

Adrenochrome i formed from A as an i termedi te product both i the THI eaction and i the EDA reaction (see D 3.6) However the manipulati e teps i the assay procedure must be different bearing i mind the instability of ad enochrome i lkaline sol tion In the EDA assay the oxidation of A i lkaline sol tion by dissol ed oxygen i controlled because as each molecul of chrome is formed it i trapped i the nascent tate by EDA to form a tabl fluorescent condensate In the THI reaction such an oxidation would be uncontrolled Instead A i oxidi ed by an added agent i mildly acidic solution to form adrenochrome which can then be tabilized i alkaline solution by a tabili ing agent which llows tautomerization of the chrome to the fluorescent THI

D 4 5 specifi ity: Hydroxyindol assays as group or broadly specific for compounds having 2-(4-hydroxyphenyl)ethylamine nucleus and thus a large number of compounds with certai structural similarities can be converted to fluorescent HIs. However ther are a number of parameters

I the HI assay schedul (see section D 4 2) e g oxidation pH use of a catalyst type of oxidant time allowed for oxidation types of reducing/stabili ing agents mi ed with NaOH nd thei concentrations time llowed for tautomeri tion the pH at which fluorescenc is recorded the time equired for maximal development of fluorescence and the optimal wav lengths (see g Laverty & Taylor 1968) The number of possible ombinations of these variables is extremely large For any compound which can form fluorescent HI the opt mal conditions ar defined by its specifi substitutions on the 2-(4-hydroxyphenyl)ethylamine nucleus. The result is i most cases HI assay with high degree of sensiti ity and specifi ity for each compound

Using the assay for A and NA the hydroxy groups on the ing (3- and 4-positions) and on the side-chai (i the 2-position) of these amines characteri the fluorophore as TH) The structural similarities of these two amines are such that when present i biological material they are generally purified i to the same eluate The N-methyl group which distinguishes A from NA influences eral parameters sufficiently to permit thei differenti I but not separate assay e the rate of oxidation t different pHs sometimes the degree of tabiliz tion achieved by different agents the time requi ed for fluorophor development and the small differences i wavelength maxima (see section D 4 2)

In THI assays for A nd NA compounds gi ing high i terference ar other I (3 4-dihydroxyphenyl)-2-amino-ethanol (i ther β-hydroxyl ted CA) derivatives g isopropyl NA (isoprenaline) (40 100%) and N-ethyl NA (66%) Other compounds gi ing moderate i terference include the α-substituted derivati es g α-methyl NA (6 9 5%) and α-ethyl NA (5 9%) (Crout 1961; Anton & Sayr 1962; Waldeck 1968; Griffiths t al 1970) A sensitive ferricyanide / / ascorbi acid type of THI assay has been developed for α-methyl NA (Waldeck 1968)

Other compounds which may gi moderate but important interference are DOPA (6 12%) (probably dihydroxyphenylserine i DOPS) α-m thyl-DOPA (17%) and α-methyl-DA (38%) especially i ferricyanide / ascorbi id assays when reading i lkaline solution (e g Pri e & Pri 1957; Anton & Sayr 1962; Carlsson & Lindqvi t 1962b; V lori al 1970; also Paper 1) maximal fluorescenc t least for DOPA being at lower wavelengths However i terference by DOPA i thi type of assay i onsiderably decreased when ecording fluorescence i acid solution (< 1%) (P i & P ice 1957) and i assays using iodine as oxidant (2.2%) (Crout 1961) DOPA appears to be a special case i tha two tructurally different fluorophores may be obtained i HI assays and thi may be rel ted to the retention or elimination of the carboxy group An assay optimi ed for DOPA of the ferri yanide / ascorbi acid type with readings i alkaline solution has been described (Kehr t l 1972; see also Valori t l 1970) and the carboxy group may be retained i the fluorophor

Derivatives lacking the hydroxy group i the ing 3-posi tion e.g M and NM giv fluorophores with similar wavelength maxima to A and NA but with ery littl (< 1%) interference

tronger oxidi ing conditions individual assays for these compounds have been described (e g Bertler Carlsson & Rosengren 1959; Carlsson & Lindqvist 1962a; Häggendal 1962a; Anton & Sayre 1966; Laverty & Taylor 1968; and Bigelow & W Il Malherbe 1968)

The l tines of derivatives lacking the side-chain hydroxy group generally give weak fluorescence in alkaline solution nd with similar wavelength maxima thus DA gives 1 4% interference (e g Bertler et al 1958; Vendsalu 1960); this interference tending to be slightly higher whe using iodine as oxidant (3 9%) (Shore & Oll 1958; Crout 1961) However in assays using a final acidification interference in the A and NA assays by DA tends to be much lower (<1%) (Price & Price 1957; Griffiths t al 1970; Valori t al 1970) because even although their fluorescence intensity may be generally increased longer development time i required and fluorescence is mitted at much shorter wav lengths than for the A and NA l tines Optimized assays for DA and related compounds have been developed (see section E 4)

The choice of purification procedure depends on which interfering compounds are lik ly to occur Alumina may be useful for separating 3-O-methyl compounds Cation exchange columns are valuable for removing non-bases especially DOPA T separate DA strongly acidic cation exchange columns are generally required

The different types of THI assays have been used for meas uring A and NA in various biological materials using most common types of purification procedure Applications include alumina purification for amines from brai (e g Chang 1964; Shellenberger & Gordon 1971) plasma (Price & Price 1957; Merrills 1963; Campuzano t l 1975) and urine (g Crout 1961; Euler & Lishajko 1961; Kahane & Vestergaard 1965) and for detailed application see Anton & Sayre (1962); solvent partitioning for brai (e g Shore & Olin 1958; Welch & Welch 1969; Miller t al 1970); CG 50 type columns for brai (e g Fleming et al 1965; Barchas t al 1972) and for blood (Renzi l et al 1970); and Dowe 50 type columns for brain (Bertler t l 1958) and blood plasma (Vendsalu 1960; Häggendal 1963a)

D 4 6 choice of assay: There are considerable difficulties i choosing which type and which of the many variants of the THI assays to use for A and NA; the results of comparative tests may differ considerably between laboratories The following are a few points for consideration

The ferricyanide / ascorbic acid assays (section D 4 2c) are probably the most commonly used being very simple to perform and operating reliably i most laboratories Sensiti ity is satisfactory for many purposes, and may be extended by using variety of approaches to assist i stabili ing the rather high blank fluorescence associ ted with the use of ascorbic acid which is an effective stabilizing agent i alkaline solution

For differentiating between similar amounts of A and NA, a ingle assay using reading at off-peak wavelengths may be used (inde of discrimination rati of A NA fluorescence is ca 5) For greater discrimination High pH" and a Low pH" assay may be used but in both assays A fluorescence tends to be greater than NA fluorescence and the index of discrimination is only slightly raised (e g to ca 15)

Using iodine / ascorbic acid i 2-component assays (section

D 4 2a), a much higher index of discrimination may be achieved (e g 300) because I the High pH* assay NA fluorescence is greater than A fluorescence In the above types of assay low interference by NA in assays for A is easier to obtain than the reverse

Fluorescence of the lutines is much mor intense in acid than I alkaline solution but their stabilization is more difficult to achieve

The iodine / EDTA / sulphite assays with readings in acid solution (section D 4 2b) provide very simpl assays with low and stable blanks However such assays may be more susceptible to purity of water and other reagents and thus show greater variations in performance from laboratory to laboratory In few instances xtremely sensitive assays for A and NA have been reported using 2-compone t assays which lso give high indexes of discrimination (g 1400) Neverthelss using iodine as oxidant highest sensitivity may not always be achieved

Using ferricyanide / mercapto-alcohol assays (section D 4 2d) comparatively intense fluorescence may be more universally obtained from both NA and A in High pH assays to provide very high sensitivity and differentiation may be based on reading t two sets of wavelengths Replacing mercapto-alcohols by thioglycollic acid provides High pH assay for NA with very low i terference from A but the NA l tine l also poorly stabilized and the procedures are best used as automated assays

Assays using sulphydryl compounds require onsiderabl care to avoid contamination and inhibition of fluorescence from these agents

Using a mercapto-alcohol l Low pH assay ery high sensitivity can be obtained from A but the low interference by NA may be variable Thi group of assays would appear to reliably offer the highest degree of sensiti ity and if the NA i terference i the Low pH assay can be standardized lso a high index of discrimination (g 100) using 2-component assays

Thus the advantages nd disadvantages of each type of assay must be weighed against the sensitivity and degree of discrimination re quired; whether measurement of one or both amines is needed and the proportions of A to NA i the tissue A number of THI assays ar now availabl which have adequate discrimination and i which sensitivity exceeds that required to ccurately measure A and NA i small volumes of normal blood plasma

PRESENT PROCEDURE for the *TRIHYDROXYINDOLE* FLUORIMETRIC ASSAY of ADRENALINE and NORADRENALINE

I I troduction: T assay A and NA in N -aqueous- HCl luate after their purification on Dowex 50 column, THI assays using ferricyanide as oxidant have been found satisfactory for the last 20 years

The simpler and more commonly used procedure i ferri cyanide / ascorbi acid assay (Bertler t l 1958) which has ecently been modified (Kehr t l 1976) When greater sensitivity is required the ferricyanide / BAL assay (Häggendal 1963a) i ofte preferred

Since most of our analyses are of NA i the CNS these assays are used with pH 6 5 oxidation and fluorescence recorded at NA wave-length maxima When values for A are lso required differentiat generally made by eading at two sets of wavelengths i High sometimes coupled with Low pH* assay (Häggendal personal

E 1 Introduction For reviews of fluorimetric assays used for dopamine (DA) (3-hydroxytyramine) I 2-(3 4-dihydroxyphenyl)ethylamine see g Carlsson (1959) Callingham & Cass (1963) *Häggendal (1966) W H Malherbe (1968)* Shannon (1971) nd Anton & Sayr (1972) Dopamine can be measured fluorimetrically using the same three principles of assays as for the other CAs A and NA (see section D 1 1) I e by nati e fluorescence condensation with EDA to yi ld fluorescent quinoxalines or oxidati e cycli tion to form fluorescent hydroxyindoles However DA was only commonly tudied after the I troduction by Carlsson & Waldeck (1958) of dihydroxyindol (DHI) assay and such assays have remained th most commonly used



E 2 NATIVE FLUORESCENCE of DA I ACID


DA I 0 1 N HCl ———— Read t 285/325 nm

The simplest of assays for DA is based on the native fluorescence of CAs t 285/325 nm whi h i optimal at pH 1 (Duggan et al 1957) However its lack of sensitivity and specifi ity and the earlier development of the mor sensiti e EDA-condensate assays made the use of thi assay generally unsuitabl for biological application *lthough it provides a ery convenient and rapid means of* monitoring the distribution of DA when establishing purification procedures (see Paper 1; also section D 2)

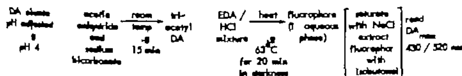
E 3 ETHYLENEDIAMINE CONDENSATE ASSAY for DAs

E 3.1 I troduction: For reviews see Weil Malherbe (1961) Udenf lend (1962) and Sharma (1971) Thi assay is based on the autoxidation of a CA to its quinone derivati and subsequent condensation with EDA to form a fluorophore Early analytical procedures used an EDA-condensate assay for measuring total CAs and a THI assay for measuring A and NA the DA being calculated from the differenc I values obtained (Weil Malherbe & Bone 1957) Later with the I troduction of HI assay for DA with a high degree of specificity (Carlsson & Waldeck 1958) this approach was generally unnecessary However using a purification procedure which separated DA from the other CAs the

high sensitivity of the EDA-condensate assay was re-utili ed with improvements i sensiti ity and specifi ity (Laverty & Sharman 1965)

E 3.2 optima: A fluorophore derived from DA can be obtained i EDA-condensate assays using the same conditions as described for A the wavelength maxima being similar (Weil Malherbe & Bone 1957a; Laverty & Sharman 1965; see lso section D 3 2)

A modified ersion of the assay i which DA was acetylated prior to the condensation reaction was i troduced by Laverty & Sharman (1965) (see also Crawford & Y tes 1970; Sharman 1971)



The DA eluate is adjusted to pH 4 prior to the formation of the tri-ac tyl ted derivative of DA which is incubated with the EDA / HCl mixture t 63°C for 20 minutes i the dark to form the fluorophore i the aqueous phase It i extracted i to isobutanol resulting i small wavelength shift and considerabl increase i fluorescence intensity i addition to being concentrated i to small olume The fluorophore appears to be photosensitiv equi ing the assay procedure to be protected from light as was found for the NA fluorophor (Laverty & Sharman 1965; see also Section D 3. 2)

E 3.3 blanks: An indication of TB values may be obtained i the same tube by ecording fluorescence lso t the wavelength maxima for the fluorophores formed from acetyl A and NA thi only being practicabl if prior purification procedure i used whi h results i the compl t separation of acetyl-DA from these compounds (Sharman 1971)

E 3.4 sensitivity: By using acetyl DA rather than DA itself for condensate formation four-fold increase i sensitivity i obtained i addition to increased specificity The ther features contributing to the high sensitivity of EDA-condensate assays are also tained i the total luate ontai ing most of the DA originally present i the biological materi l may be used i the sampl tube for conversion to the fluorophore which i then concentrated into ery small volume of organi solvent Furthermore TB may be obtained i the sam tube Sharman (1971) *subdivided the eluate using portions for S / TB and ISt* Thus sensiti ity was of the order of 2 ng per cuvette ml and since the total DA content may be transferred to the cuvette volume (2 ml) sensitivity of 10^{-9} g DA i tissue may be measured (see Laverty & Sharman 1965)

E 3 5 chemistry: The chemical basis of the EDA-condensate assay for DA common with other catechol compounds involves the oxidation quinone derivative which i alkaline solution readil

EDA to form a tabl polycycl fluorophore The chemistry of the reaction of DA with EDA may well be similar to that for A (as proposed I Section D 3.6); since the lkyl lde-chal is retained I forming the fluorophore which has simll optical characteristics to that formed from A (non-acetylated) (Laverty & Sharman 1965)

When the CAs were acetylated prior to assay the wavelength maxima for DA (430/520 nm) become different from that of A (A and NA-max both being 420/490 nm) During the incubation eaction hydrolysl of the tri-acetyl-CAs will occur to form the N-acetyl-CAs (see Crawford & Y hes 1970) The DA side-chai is probably retained and cyclized during the eaction (see Harley-Mason & Laird 1959) For A which has a hydroxy group on the carbon tom adjacent to th benzene ing the presence of the N-acetyl group may result I a imilar reaction to that of NA I which case the fluorophor formed I both cases will be that derived from catechol (as discussed i Section D 3 6)

E 3.5 specifi ity: The EDA-condensate assay I broadly specifi for catechol compounds. When using the procedur for DA greater specificity is obtained by final extraction of the fluorophores into an organi sol ent However the fluorophores of ther CAs e g A and NA will lso be extracted as will those of non-basi catechol compounds I whi h the alkyl ide-chain I cast off during th condensation eaction to form the fluorophore of catechol itself g DOMA. The solvent extraction largely excludes however those non-basic catechols I which the alkyl side-chai may be retained I the formation of the fluorophore g DOPA and DOPAC (see Section D 3.6) When assaying the DA base a number of catechols may therefore I terfere; A α-methyl-DA N-methyl DA and probably some ther CAs which etai their side--chains hav wavelength maxima imilar to those of DA (Kögl et al 1957; Laverty & Sharman 1965) Those catechols whi h lose thei side-chains to form the fluorophore of catechol g NA and DOMA may be differentiated by utilizing the differen I thei wavelength maxima

When assaying DA after its preliminary acety specifi ity is increased The most important i terfering probably α-methyl-DA its fluorophore having imilar wavel maxima to that of DA; but an increased number of fluorophores with wav length maxima different from those A and isoprenaline (Laverty & Sharman 1965) this possibly to the N-acetyl substituent inducing the elimination of the to form the fluorophore of catechol

Nevertheless suitabl purification I most I obtaining adequate specifi ity The earlier wide-spread use for purifying CAs had to large extent endered the assay unsuitabl for assaying DA because both procedures dependent on the catechol group for specificity and even t of a weak cation xchange procedure for emoving non-bases leaves the possibilities of i terference by other CAs The of DA from A and NA by th use of Dowex 50-type

(Bertler et al 1958; Sharman 1971) (or of their acetylated derivati es by paper chromatography Laverty & Sharman 1965; Crawford & Yates 1970) coupled with the innovation of ac tyl ting DA prior to its EDA-condensate assay (Laverty & Sharman 1965) has provided an analytical procedure for DA with a high degree of specifi ity as well as sensitivity This has proved useful for measuring DA i ery discrete areas of CNS and other tissues (see Sharma 1971)

E 4 DIHYDROXYINDOLE ASSAY for DA:

E 4.1 Introduction For review of these assays see g Carlsson (1959) Callingham & Cass (1963) Weil Malherbe (1968) Udenfriend (1969) Sharman (1971) and Anton & Sayre (1972) Thi assay i based on the controlled oxidation of DA to its chrome derivativ which is believed to undergo molecular rearrangement i a trongly alkaline solution i the presence of a suitabl reducing and tabilizing agent i imilar manner to A and NA to form a DHI which gi es trong and tabl fluorescence i acid solution Thi assay was i troduced and used for measuring DA i brain by Carlsson & Waldeck (1958)

Carlsson & Waldeck (1958) had noted the observation by Duliére & Raper (1930) that DHI could be formed from DA. In the early HI assays for A and NA used between 1949 and 1962 ascorbi acid was used to tabili the fluorescent i tines in trong alkali Under such conditions the fluorescenc developed from DA had similar wavelengths but was very weak ompared with that from A and NA However Carlsson & W ldeck found that by acidifying the sol tion after fluorophor formation ery i tense fluorescence slowly developed t wavelengths much lower than those of A and NA: also that ascorbi cid interfered with fluorescence at these low wavelengths but that it could be replaced by sodium sulphite.

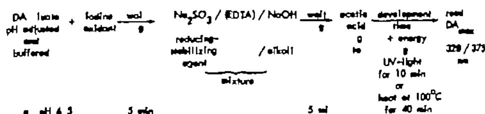
The HI assay for DA i troduced by Carlsson & Waldeck (1958) used iodine as oxidant and sulphite as reducing / tabilizing agent with a final acidification. It provided not only the first and still the most commonly used type of fluorimetri assay for DA which has high degree of specifi ity as well as sensitivity; but i turn it was used for developing imilar types of HI assays for A and NA (e g Chang 1964; Laverty & Taylor 1968) i which high fluorescenc i tensity was combined with lower and more tabl blanks and higher powers of discrimination i differential assays for A and NA (see section D 4 2b)

Subsequently iodine has been the most commonly used oxidant i DHI assays for DA although manganese dioxide has been used (Uuspaa 1963) and greater sensitivity has been obtained using periodate (Anton & Sayre 1964) and more recently ferricyanide (see Paper III and section E 5)

E 4 2 optima general Many of the general considerations for THI assays of A and NA also apply to these DHI assays for DA (see section D 4 2"

E 4 2 IODINE as oxidant plus SULPHITE as reducing / stabilizing agents

Thi type of assay i troduced by Carlsson & Waldeck (1958) and later studied in detail by Laverty & Taylor (1968) is widely used with minor modifications (e g Drujan Sourkes Layne & Murphy 1959; McGeer & McGeer 1962; W lch & W lch 1969)



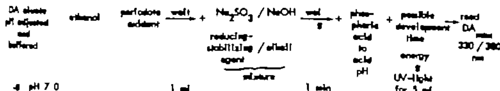
In this type of assay the eluate i carefully adjusted with e g K_2CO_3 to a fi ed pH generally between 6 and 7; and buffered usually with cetat or phosphate buffer (g Laverty & Taylor 1968) The optimal amount of iodine is then added and allowed to act for a fi ed time e g 5 mi tes before adding the optimal amounts of ulphite and NaOH as a mi tur (EDTA has also sometimes been incorporated but its use does not appear to be as essential as i the equivalent type of assay used fo A and NA; however it may be useful if alcoholic iodine solutions used see section D 4 2b) (g. Carlsson & Waldeck 1958; Laverty & Taylor 1968; Ansell & Beeson 1968) After llowing a fi ed tim gener lly between 3 and 5 minutes for tautomerization the sol tion i finally acidified usually with ac tic acid to the optimal pH g 4 4 Thi esults i a hypsochromic wavelength shift from 380/460 nm i alk li to 320/375 nm i acid with concomitant increase i fluorescenc i tensity and stability (see Carlsson & Waldeck 1958; Laverty & Taylor 1968) Development of fluorescence is low at oom temperature equi ing between 45 minutes (g Drujan Sourkes Layne & Murphy 1959) and 20 hours (Bischoff & Torres 1962) but can be accelerated by using energy g. UV light for 10 minutes (Carlsson & Waldeck 1958) or heating i water bath e g at 100°C for 40 minutes (Laverty & Taylor 1968; Wel h & W lch 1969 se also Paper III and section E 5 2) The fluorescenc is then ery table for several hours (Carlsson & Waldeck 1958, Welch & Welch 1969)

An automated ersion of this type of assay has been developed (Waldmeier & Maître 1973)

E 4 2b PERIODATE as oxidant plus SULPHITE as reducing / tabilizing agents

Thi type of assay was i troduced by Anton & Sayre (1964) and later tudied by W ll Malherbe (1968)

Periodat is used for oxidation at g. pH 7 0 and if ethanol is present final fluorescenc is increased Oxidation time is restricted to 1 minut and one luate is assayed at time After addition of sulphit and NaOH as a mi ture fi ed time of up to 4 minutes may be allowed for tautomeriz tion A final acidification



I mad using either mi ture of itrat and phosphoric acid (plus HCl) (Anton & Sayr 1964) or phosphori acid ion (Weil Malherbe 1968) Fluorescen e i recorded immediately by Anton &Sayr (1964) but increased i tensity after UV irradi tion for 5 minutes was reported by Weil Malherbe (1968) who ecorded fluorescenc fter waiting for 1 hour

E 4 2 FERRICYANIDE as oxidant plus SULPHITE as reducing / stabilizing agents

This type of assay which was ecently i troduced has advantages of greater sensitivity and convenience (see section E 5)

E 4 3 blanks: i these HI assays two principl types of TB procedure are used; faded samples (TBf) and unoxidized samples (TBu) The various ways of preparing these blanks are essentially the same as in the A and NA assays (see section D 4 3) However the TBu obtained by premixing ll assay reagents prior to addition of the acid eluate whi h was found to be so suitabl i th A and NA assays has rarely been used for DA assays (see however E 5.3)

E 4 4 chemistry: The hemical basis of hydroxyindol assays has been discussed above (see section D 4.4) For DA the sequenc of eactions whereby DA is converted by oxidative cyclization and i tramolecular rearrangement to 5 6-dihydroxyindol as established by Dulière and Raper (1930) is form l ted bov (see section D 4 4)

In most types of HI assay for DA some for NA and one for A the fluorescence develops only slowly after the HI has been formed (see section D 4 2 and E 4 2). One possibl explanation of this phenomenon is that the HI formed by tautomerization of the chrome derivative has to exi t in specifi ionic form for fluorescence to occur Possibly the conversion to thi ionic form is influenced by number of f ctors including the type of substitutions on the HI nucleus and the edox potential established i the solution at parti ular pH (see g Laverty & Taylor 1968) Such conversion to the required ioni form may be accelerated by the use of intense sources of energy such as heat or UV irradiation

E 4 5 specifi ity: Although HI assays as group are broadly specifi for compounds having 2-(4-hydroxyphenyl) thylamine nucleus (see secti D 4 5) specifi substitutions i this nucleus can result i relatively specifi assays for indi idual compounds. Using the assay for D hydroxy groups on the ring (3- and 4-positions) and the thylam side-chai characterize the fluorophore as DHI as the basis ly sensitive and specifi assay for DA. Parameters whi h

contribute to the specifi ity of the assay include the conditions of oxidation and tabilization and mor especially the acid pH t which fluorescence I recorded the longer time of development of fluorescence and the onsiderably shorter wavelength maxima

I the DHI assay for DA compounds gi ing I terference are certai other 2(3 4-dihydroxyphenyl) thylamine derivati es The most important of these is DOPA which I equimolar amounts may give the same order of fluorescenc as DA (70 – 140%) at similar wavelength maxima thi may result from elimination of the carboxy group Other I terfering compounds include α-methyl-DOPA (3% I terference) α-methyl-DA (11%) and N-methyl DA (epinine) (19%) (Anton & Sayre 1964; Waldm ier & Maître 1973) A similar type of assay has been developed for DOPA (Fahn P asad & Delesi 1972) and N-methyl-DA may be assayed using a THI ferricyanide/ascorbic acid type procedure (see section D 4 2c) (Carlsson & Lindqvist 1962 b)

Derivati es lacking a hydroxy group in the ing 3-posi- tion (e g 3-MT) hav fluorophores with similar wavelengths and may giv moderate (9 – 18%) I terference (Waldmeier & Maître 1973) Using stronger oxidi ing conditions, indi idual assays for 3-MT have been described (Carlsson & Waldeck 1964; Messiha & Raval 1973) M och NM giv very low I terference in this assay (<0 05 2%) (Anton & Sayr 1964; Waldmeier & Maître 1973) (for specifi assays of M and NM see section D 4 5)

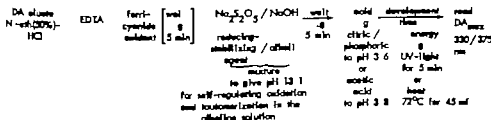
Deriv tives with a hydroxy substitution I the ide-chai (2-position I β-position) giv weak I terferenc g A and NA (<0 05%) (Anton & Sayre 1964) I acid sol tion t wavelengths used for the DA fluorophor and the conditions used for oxidation and tautomerization do not lways lead to effecti ely stabilized A and NA fluorophores For further considerations of specificity see section E Paper III

The choice of purification procedure depends upon which possibl I terfering compounds ar present Thus cation exchange columns are useful for separating DOPA nd other non-bases from DA *and alumina for separating 3-O-methylated derivatives from DA.* T separate A nd NA from DA strongly acidi cation exchange columns are generally equi ed However it was for eluates from these latter that the DHI assays for DA wer introduced to measur DA I brai (Carlsson & Waldeck 1958; Carlsson & Lindqvi t 1962 b; see also Carlsson 1959 a) although they hav been mainly used following alumina purification to measur DA I brai (Anton & Sayre 1964; Karasawa t al 1975) spinal cord (McGeer & McGeer 1962) and urine (Bischoff & Torres 1962)

E 5 PRESENT PROCEDURE for the DIHYDROXYINDOLE FLUORIMETRIC ASSAY of DOPAMINE

E 5 1 Introduction A new type of DHI fluorimetri assay for DA which has been applied to the N-ethanolic(50%)-HCl from the Dowex 50 purifica- tion procedure (see section A 4) is described I Paper III

I the assay oxidation of DA by ferricyanide and subsequent tautomerization both occur in the same trongly lkaline solution I the presence of sulphite as reducing and stabill ing agent A final cidificat on l mad and development time is requi ed for maximal fluorescence The assay I simpl and ery eliabl and the self-regul ting nature of the reaction mak the assay very suitabl for the routine simultaneous analysis of large numbers of samples Blank fluorescenc tends to be low and tabl This is probably the most sensitiv version of the DHI assays which re availabl for DA.



In the The procedure the DA eluate is added after mixing the assay reagents

E 5.2 optimax The luate for whi h this assay was optimized is N-ethanol (50%)-HCl The EDTA is added to prevent the formation of precipitate if heat is used to ac elerate the development time for the fluorophore I ac tate sol tion The optimal amount of ferricyanide is added and time I terval is llowed negligibl oxidation of DA occurs at thi tage I N HCl The presence of 25 50% ethanol I the eluate increased fluorescenc by 30% apparently by influencing the oxidation or tautomerization eactions sinc if the thanol was added after the alkali fluorescence increased by only 10%. The optimal amounts of sulphite and N OH were added as mi ture so that oxidation nd tautomerization could occur Sodium sulphite or metabisulphite were equally suitabl as educing agents Although the pH of the reaction was shown to be rather critical t pH 13 1 (ca 0 1 N NaOH) the assay was found to be highly reproducibl after mixing the 1 N HCl with ca 3 N NaOH without the presenc of buffer and reproducibility should be at least as good when using more weakly acidic eluates. The amount of NaOH must be adjusted to produc the optimal pH of 13 1 (Paper III) when adapting the assay to different DA eluates g. from lumina olumns (Karasawa Furukawa Yoshida and Shimizu 1975) The oxidation and tautomerization occurred rapidly l less than 20 seconds. The DHI appeared to be stabl I the alkaline sol tion since time I terval of t least 15 minutes could elapse without effecting the results. However fixed time was chosen for this and the previous interval appropri te to the number of samples being assayed to avoid I troducing unnecessary variables

For acidification to an optimal pH of about 3.6 either acetic acid or itric/phosphori acid could be used The pH was tained more reproducibly with the former acid but 15% higher fl escnec I tensity could be obtained using the l tter acid mi t

Fluorescence developed slowly but could be accelerated by applying energy Near-maximal fluorescence could be obtained i a variety of ways g by heating at 72°C for 45 minutes or irradiating with UV light Several factors could influence the conditions required for development of fluorescence: e g using UV lamp peak emission 254 nm 7 minutes was equired fter acidification with citric/phosphoric acid but only 5 minutes after aceti acid i e if the reaction was carried out i quartz test-tubes but if pyrex glass tubes were used then 15 minutes wer required when using acetic acid: when using heat temperatur below the boiling poi t of ethanol was preferred g 72°C for 45 minutes to obtai more reproducibl results These observ tions ar i teresting i iew of the conflicting reports concerning the most suitable ways of developing maximal fluorescenc in the iodine/sulphite DHI assays for DA (see section E 4 2) Once developed fluorescence was stable for t least 4 hours

An automated ersion of thi assay has been described (Prasad Fahn & Isgreen 1975)

E 5.3 bl nks: *The most suitable TB procedur was found to be a TBu i* which ll assay reagents were mi ed i the normal order before finally adding the portion of acid eluate This TBu" and TBf" i which the NaOH was added alone followed by the sulphite after the final acidification when tested on pure sol tions of DA were found to be essentially the same as RB independantly of the amount of DA present (up to 5 μg tested); whereas other types of TBu (see section D 4 3) gave increased readings with increasing DA concentration When assaying eluates from blank Dowex 50 columns the PBf" tended to be slightly too low and the PBu too high as ompared with PS due to slight i terf rence by material derived from the resi (for discussion see Häggendal 1962b) The use of the TBu was therefore likely to lead to slight under estimation of DA i the Dowex 50 eluates ca 1 ng DA per column

The TBu procedure was preferred and when applied to eluates deri ed from brai extracts of rats treated to depl te the brain of its DA content a concentration of ca 5 ng per g was obtained i less than 1% of the DA content of normal brai

TBu prepared as described using pre-mixed assay reagents before adding the acid eluate have previously been shown to be very suitable for use in THI assays for A and NA (e g Chang 1964; O'Hanlon t al 1970; see section D 4 3)

One factor i particular which led to considerable vari tions (up to 300%) in the magnitude of the RB was the purity of the thanol and of several brands and grades tested the Merck spectroscopic grad 95% gav the i west blanks (see Paper III) Subsequently i another laboratory much less expensive 99% industrial grade of thanol was availabl which gave ery high "RB ; but after dil ting with water and subjecting it t e simpl eflux distill tion and discarding the first nd last 5% of the di till te the ca 96% ethanol (azeotropi mi ture) so obtained gave RB even slightly lower than the best results previously obtained

E 5.4 chemistry: The nature of the chemical eactions undergone by DA in this assay are presumed to be those typical of other DHI assays for DA (see section E 4 4) A unique feature of this assay is that not only the tautomerization of the DA chrome but also the oxidation of DA to its chrome derivative occur i the same strongly alkaline solution (here bearing certai resemblances to the fluorimetric assays used for A in the early 1940 s see section D 4 1) in the presence of a educ ing agent to form a stabl HI fluorophore This is possible presumably because of the redox potential established by the particular combination of oxidizing and reducing agents used for DA in the section Oxidation of DA by ferricyanide i the strongly alkaline solution occurs almost instantaneously whereas reduction of the ferricyanide by sulphite equi es several seconds thus llowing time for DA to be oxidized and i addition the sulphite is lready present to stabilize the DA – chrome i alkaline solution as i other DHI assays for DA (see section E 4 2) With many ther combinations of oxidizing and reducing agents it would appear that the oxidizing agent is reduced too rapidly for oxidation of DA to occur

E 5.5 specifi ity: Thi type of DHI assay for DA tends to be more specific than those using iodine as oxidant and as specific as that using periodate Most of the compounds found to give more than 2.5% equimolar i terference i the assay re compl tely separated from DA using the Dowex 50 purification procedure including DOPA 3-methoxytyrosine and 6-hydroxydopamine The notabl exceptions are α-methyl-DA which gives ca 8% i terference i the assay and overall procedures and 3-MT which gives somewhat variabl interference of 12 20% i the assay and up to 20% i the Dowex 50 eluate gi ing an overall interference of ca 4%. The use of differential assay for distinguishing between DA and DOPA (ca. 70% interference) which has been incorporated i the periodate DHI assay for DA DOPA interference of ca 143%) (Anton & Sayre 1964) was unnecessary because DOPA i compl tely separated from DA on the Dowex 50 columns (Paper I see also Weil Malherbe 1968)

E 5.6 sensitivity: In thi assay sensitivity expressed in the popular manner of twice the RB is equivalent to 3.7 ng DA per cuvette volume However smaller amounts of DA could be assayed since one meter unit above the RB was equivalent to 0 08 ng DA per cuvette volume with the RB readings being ery stabl a reading of 5 meter units above the RB (which is 2 S.D of the mean RB reading) i equivalent to 0 4 ng DA per cuvette volume Using 1 ml out of a 3.5 ml eluate in the assay and adding only 0 6 ml of assay reagents amounts of DA of the order of 10^{-9} g i tissue may be measured

E 5 7 general comments: This assay procedure for DA has been used successfully in several other laboratories throughout the world where its sensitivity compared with other DHI assays has been confirmed (e.g. Kurosawa et l 1975; Prasad et al 1975).

The assay was developed for application N-eth (50%) HCl luate obtained from the new Dowex

cedure (see Paper III) Previously a N HCl eluate had been obtained (Bertler et al 1958) with a DHI assay using iodine oxidation at pH 6 5 (Carlsson & Waldeck 1958) or t pH 5 4 (Carlsson & Lindqvist 1962 b) In preliminary experiments for assaying DA 1 ml of the N-ethanolic (50%) HCl eluate was adjusted to pH 5 4 and (with appropriate adjustments for eluate volume) this iodine/sulphite DHI assay was applied; but because very variabl results were obtained various parameters were checked Surprisingly ferricyanide oxidation of DA was found to result in fluorescence intensity approaching that obtained using iodine t pH 5 4; and even more unexpectedly fluorescence was found to increase as the adjusted pH of the eluate was lowered Its intensity became greater than could be obtained using iodine oxidation and reached a maximum when applying ferricyanide to DA in N HCl The observed optimal pH for oxidation was really the result of the strongly acid eluate neutralizing the NaOH concentration towards pH 13 for tautomerization, at which pH the oxidation by ferricyanide was also occurring (Thus, when establishing the optimal pH for oxidation i conventional HI assays, care should be taken to maintain a constant pH for tautomerization when testing solutions of various pHs for oxidation) The realization that both oxidation and tautomerization were occurring at pH 13 was checked by preparing solutions of various normalities of NaOH containing sulphite befor adding DA and the ferricyanide Near maximal fluorescence was till obtained in thi reverse order assay with ca 0 1 N NaOH sol tion

In the ferricyanide/sulphite DHI assay results were very reproducibl nd uninfluenced by certain climatic conditions Thus, this new assay was developed during hot summer conditions which we find renders the iodine/sulphite DHI assay unusabl ; partly because the high ambient temperatures increased the amount of spontaneous oxidation of DA, especially during the pH adjustment resulting in high blank values (Improved results were obtained i this assay by changing to a TBu i which all the reagents were premixed before adding the a id eluate see e g section E 4 3 and E 5 3) The satisfactory performance of the new ferricyanide/sulphite DHI assay is probably related: partly to the considerable heat generated upon the mixing of strong NaOH and HCl solutions; partly to omitting titration of the acid luate to a near-neutral pH; and partly to the use of a TBu with premixed assay reagents plus acid eluate

Iodine is the most commonly used oxidant i DHI assays for DA but these assays are known to not function satisfactorily i all laboratories (e.g see Sharman 1971) It is thus useful to have a different type of oxidant available for the DHI assays e g the ferricyanide/ /sulphite assay which has been found to function satisfactorily in Dr Sharman s laboratories (personal communication)

The numerous advantages of this assay procedure have proved very valuable for the outine analysi of large numbers of DA samples; nd coupled with the Dowex 50 procedure provides a high degree of specificity and sensitivity It has been used for measuring the small amounts of DA in peripheral tissues such as heart and saliva

gland (Snider t al 1973) Also it has enabled a detailed study to be made of the changes in DA concentration following spinal cord transection (Magnusson 1973) after the method had been shown to be suitabl for measuring DA i this tissue (see Paper III)

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SUPPLEMENTUM 452

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A Tribute to Bengt Falck

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A Tribute to Bengt Falck

Proceedings from a symposium held January 14 1977
at the Department of Histology University of Lund
on the occasion of Bengt Falck's 50th birthday

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CONTENTS

A TRIBUTE TO BENGT FALCK

On January 16 1977 Professor Bengt Falck celebrated his fiftieth birthday

It is superfluous to describe the details of Bengt Falck fundamental contributions to modern medical science with important implications on wide areas of neurobiological and endocrinological research Falck name is primarily connected with the histochemical method for cellular demonstration of certain biogenic amines which he developed together with the late Professor Nils-Åke Hillarp Not to forget his classical M D thesis on microtransplantation of ovarian endocrine cell systems in which Bengt Falck elegantly demonstrated that th production of estrogen is depending on an interplay between theca interna gland cells or interstitial cells and corpus luteum cells or granulosa cells

The fluorescence histochemical method of Falck and Hillarp is based on the introduction of new fundamental technical principl namely that the chemical reactions i the tissue proceed under dry circumstances including the use of reagents i gaseous form so that diffusion of the reacting substances is maximally prevented which results in very precise cellular localization of the amine The method thus allowed for the first time the visualization of i traneuronal transmitter substances The comprehensive analysis of the chemical background of the method distinguishes it from most other histochemical methods inasmuch as it is known in detail how the reaction proceeds when formaldehyde condenses with the monoamines to form fluorophores which can be observed in the cells by fluorescence microscopy Moreover unique degree of sensitivity has been reached because the method is based on fluorescence reaction and not conventional staining procedure The method is with certain prerequisites quantitative and can thus be used for highly sensitive micro analysis of functional changes of the monoamines withi individual cell

Bengt Falck tarted to work i Nils-Åke Hillarp laboratory at the Department of Histology i Lund almost 30 years ago In the late 1950 Hillarp introduced the line of monoamine histochemistry into the research of his laboratory The motivation for this was obvious In the brai the presence and regional distribution of noradrenaline and 5-hydroxytryptamine had been known for some years from the work of von Euler Holtz Vogt Amin Page Carlsson and thei coworkers and recently Bertler and Rosengren in Lund had reported

the occurrence of large amounts of dopamine i the caudate nucleus Although the view that these monoamines act as transmitters gained strength many found it hard to accept transmitter function for 5-hydroxytryptamine and dopamine Thus cellular localization of these amines in brai was required to make the discussion of thei functions meaningful Moreover von Euler s concept of noradrenaline being the sympatheti transmitter i the periphery had been questioned by Burn and his group who put forward the hypothesis that *peripheral noradrenalin is stored in and released from peripheral cell perhaps chromaffin* cells innervated by cholinergic nerves

The idea to prevent dislocation of the monoamines from their cell lar stores crystallized from a series of rather disappointing studies where Falck i collaboration with Alf Torp attempted to transform Ehrlén trihydroxyindol method for histochemical purposes The first trials with gaseous reagents such as iodine and ammonia vapors as well as with thylen-diamine were not successful However exposure of sections from adrenal medulla to formaldehyd vapor generated from saturated formaldehyde solution induced very intense fluorescence in the noradrenaline cells That became the deciding turning point; ll efforts in the laboratory were then directed towards devising *histochemical formaldehyde* method Professor Hillarp at that time working i Arvid Carlsson laboratory in Göteborg under a MRC grant travelled to Lund to discuss the further work and Bengt Falck tells us about the particular exciting day i August 1961

"That is day I will never forget as long as I live Hillarp who had great experience in preparing whole-mounts of thin tissue-sheets from his work on the autonomi ground plexus, suggested that we should try to expose rat iris to dry formaldehyde gas He prepared an iris It was llowed to dry in air for short time and was then exposed to dry formaldehyde gas for 1 hour at +80° C The slide was put under the microscope with no great expectations but the picture surpassed our wildest hopes There was the adrenergic ground plexus exactly as Hillarp had described it many years earlier except that this time the nerves were not stained with methylene-blue but th transmitter itself showed up i form of its fluorescent derivative There were lso th weakly fluorescent preterminals and the strongly fluorescent varicose terminals — but no chromaffin cells — thus confirming the concept of von Euler on the localization and distribution of noradrenaline i the adrenergic neuron and excluding the hypothesis that postganglionic cholinergic fibres liberate noradrenalin from store around the ympatheti nerve ending W telephoned to Ulf von Euler and told him what we had just seen in the microscope not that I believe he had for a moment had any doubts nonetheless it felt good to give him thi information and he appreciated it Then we went home to celebrate and to dream about the future Our work for the next few months was



characterized by trials to standardize the method paralleled with ttempts to harvest as much information as possible about the cellular localization f the monoamines and in 1962 the first reports on peripheral and central monoamine stores appeared

Th Falck-Hillarp fluorescence method has meant breakthrough i several research areas (1) It has formed the foundation for complete and detailed mapping of the entire peripheral sympatheti nervous system and by the high precision of the method it has given very ignificant contributions to the solution of several problems of purely functional character (2) In the central nervous system it has been possibl to use the method for final proof for the transmitter functions of several ami es and it has now been possibl to map and study in detail very extensive systems of aminergi neurons Important contributions have

been offered for the understanding of endocrine control mechanisms in the hypothalamus for pharmacological and functional aspects on the extrapyramidal system for regeneration and plasticity of central neurons etc etc (3) It has been possibl to detect previously unknown occurrence of catecholamines and serotoni in several endocrine cell systems forming the base for a number of functional investigations (4) The discovery that primitive nerve systems i lower organisms work with catecholamines and serotonin as transmitters has opened new possibilities for studies of evolutionary mechanisms in the nervous system And (5) the high sensitivity and great specificity of the method has made it possibl to use it as a tool i an extremely broad analysis of the action mechanisms of different drugs primarily those which are of interest in modern psychopharmacology

F lck contributions have been exploited i different contexts both within Sweden and broad where he has been engaged in several expert and specialist panels H has been awarded the Florman Price by the Royal Swedish Academy of Sciences 1966 the Axel Hirsch Price of Karolinska Institutet and the Purkyne Medal by the Czech Academy of Sciences

Bengt Falck is Chairman of the Institute of Anatomy and Histology at the University of Lund He has unique ability to initiate stimulate fascinate and encourage His honesty and his loyalty towards colleagues and friends is absolute Against the background of these qualities Bengt Falck has at the Department of Histology created an extremely active research team which during the fifteen years the fluorescenc method has existed has produced more than 750 scientific reports Th activity beautifully expresses the position of modern histology i Sweden dynami medical cell biology bridging tructure and function

As manifestation of our admiration of Bengt Falck as scientist and friend and to celebrate his birthday a symposium was held at the Department of Histology in Lund on January 14 Invitations were sent to ll amine histochemists in Sweden present and former research tudents and colleagues of the Department including foreign guest scientists currently working i the team The conference during which fourteen papers were presented was ttended by aminologists representing variety of fields: anatomy histology zoology physiology biochemistry pharmacology surgery eurosurgery neurology ophthalmology pediatrics gynecology and dermatology More than eighty friends participants in the Symposium as well as the staff and personnel from the Institut celebrated Bengt Falck during the conference dinner The Symposium day became an expression of deep feeling of gratitude for Bengt Falck self sacrificing work to secure the continuous vitality and friendly atmosphere that are so unique for the Lund Amine Team

Christer Owman Anders Björklund

FOREWORD

The Editors of the present volume on "Current Research on the Histochemistry and Function of Biogenic Amines" have kindly asked me to write Foreword The Proceedings of this Symposium bear witness of the wide use the fluorescence histochemical technique has won in few years It is rightly considered as fundamental contribution which has formed new basi for the lucidation of the anatomy and histology of the monoaminergi systems In addition it has greatly assisted i the clarification of mechanisms involved in the synthesis, storage uptake and release f the amines by which multitude of neurons and other cell exert their physiological actions

A better basis for histochemical reaction than the fluorescence xerted under certain conditions by the actual transmitter substance itself could hardly be found Of earlier attempts in thi direction those of Olavi Eränkö in Helsinki 1955 seem to have been the most successful By treatment of adrenal medullary sections with formalin he observed brilliant fluorescence ttributable to catecholamines in the first place noradrenaline Fluorescence of catecholamines in adrenal medullary cell could lso be produced by ther methods but the noradrenaline stored i the adrenergic nerves for long time resisted the attempts t make them isible by histochemical methods The systematic work of the histology group in Lund finally brought success by utilizing the formaldehyde reaction under such conditions that the catecholamines did not diffuse from their natural cell lor ties In the first announcement of this achievement by Falck and Torp in 1962 the fluorescent network of the adrenergic fibres in the rat iris appearing after exposure of dried tissue mounts to formaldehyde vapour was described The first ight of the plexus of nerve fibres in the entire iris must have held surprising beauty for the microscopists

The results were the outcome of systematic studies long different lines, and could be rapidly exploited by research group whose members were fully aware of the ignificance of the findings Particularly interesting was the observation that the longated swellings in the adrenergic nerve terminals, described by Hillarp already in 1946, stood out with brilliance in the histochemical preparations whereas the intervening parts of the terminal axons showed only faint fluorescence making them to appear like string of pearls Thi was an indica-

tion that these varicosities contained the transmitter in high concentration an observation which was in agreement with our earlier conclusions about its distribution in the nerves based on determinations of the relative amounts of noradrenaline in the whol organ and in its adrenergic nerve bundles Further xperiments by the research group in Lund proved the existence of fluorescent fibres in all organs supplied with adrenergi nerves.

The new method to isualize specifically the adrenergi nerve system in its finest ramifications should naturally evoke intense interest in many laboratories engaged in studies of adrenergi mechanisms Moreover it created at once a wide field for detailed studies — both anatomical and functional — with a iew to gai ing more insight i the processes of adrenergi neurotransmission As result of this large number of doctoral dissertations and scientifi papers have emerged primarily from the laboratories which were originally engaged in this research

Evidence soon accumulated to show beyond doubt that the fluorescence i the neurons was due to the presence of the neurotransmitter itself T a certain xtent at least the degree of fluorescence lso afforded quantitative estimation of the amount or concentration of transmitter present even if some caution in this respect has to be xercised The ability of certain neurons to take up amines or their precursors could lso be utilized when the natural fluorescence was weak thus making the monoaminergic neurons more clearly isible in the microscope The localization of the transmitter in the neuron as well as its disappearance and reappearance in different parts of the neurons, could also be followed in way that had not previously been possible Thus the technique lent itself admirably to studies of the distribution of the transmitte after various measures designed to alter the axoplasmic flow By such techniques the transport of storage vesicles along the axon could be shown and earlier assumptions of centrifugal transport of the transmitter by the axoplasmi flow could be proven The striking variations in adrenergi nerve supply from the few fibres in the testi le to the abundant nerve net in the vas deferens form valuable complement to the chemical analyses of the transmitter in the organs Another finding which merits spe ial consideration i the demonstration of axon-like outgrowths from chromaffin cells transplanted to the anterior chamber of the eye

Even if the fluorescence technique has supplied secure foundation for the knowledge of the peripheral sympatheti system, its importance for the elucidation of monoaminergic systems in the central nervous system may be regarded as even more far-reaching Although it was known previously that the brai contained both noradrenaline adrenaline dopamine and 5-hydroxytryptamine the fluorescence technique made it possible to establish the localization and course of the fibre bundles, many associated with previously unknown pathways

which utilized these transmitters Research in this area is steadily growing, providing basis for the understanding of the physiology and pathophysiology of numerous facets of higher nervous functions

Differentiation of the fluorescence by spectrofluorometri and other methods has also proved of great value in the analysis of transmitter substances occurring in non-mammal and in evertebrates One example of the possibilitie afforded by the difference in the spectral characteristics of the fluorophores I the demonstration of dopamine cells and neurons in various tissues

Only few of the striking results obtained by the histochemical fluorescence technique have been briefly touched upon in this introductory survey; but enough, I think to show that it has opened up new and fruitful avenues f research I remarkably brief time by the co-ordinated efforts of large number of skilled and devoted research workers, clearer picture and better understanding of basic neurobiological and endocrine processes have been arrived at Some of these may in the future prove to be of still greater importance than is now realized

The development of the fluorescence histochemical technique by Falck, Hillarp and their associates represents beautiful example of successful outcome by joint efforts at favourabl time

Ulf S von E ler

Section I

CENTRAL AMINE NEURON SYSTEMS

THE USE OF MAGNESIUM IONS FOR SENSITIVE VISUALIZATION OF CATECHOLAMINES AND SEROTONIN IN THE CNS

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Several different methods are currently used for the visualization of catecholamines (CA) and indolamines (IA) in nervous tissue The most commonly used procedure is that of Falck and Hillarp (1 2) using formaldehyde (FA) treated freeze-dried tissue and paraffin embedding Later alternative procedures based on Vibratome sectioning (3 4) or cryostat sectioning (5 6) have been introduced for more rapid results Among these methods the Vibratome procedures and particularly the glyoxylic acid (GA)-Vibratome procedure are learly the most sensitive ones

With the introduction of the Vibratome methods it became lear that the original Falck Hillarp method had an unsufficient sensitivity for visualization of many CA fibre systems However ince the freeze-drying-paraffin embedding technique has several practical advantages it was i our opinion of great interest to find ways to increase the sensitivity of thi procedure to level comparable to that of the Vibratome methods Our basic assumption was that certai metal ions such as magnesium ions which re known to inhibit the release of transmitter substances would improve the isualization of CA and IA neurons by preventing the release of the amines during the histochemical processing Our results show that perfusion of the animal with solution contai ing FA and GA in combination with very high concentration of $MgSO_4$ gives striking improvement of the visualization of CA and — to lesser degree — serotoni neurons in freeze-dried paraffin-embedded tissue (7) Furthermore we have found that magnesium can be used to improve both the cryostat and the FA-Vibratome procedures for CA visualization (8)

For freeze-drying and paraffin embedding the best results were obtained after perfusion first with 150 ml of an ice-cold acid phosphate buffer (pH 4.5) followed by 150 ml of an ice-cold solution containing 2 % GA 0.5 % FA and 40 g $MgSO_4 \times 7H_2O$ (The acid pre-perfusion improves the general morphology of the tissue sections) The tissue was then freeze-dried FA-vapour treated and paraffin embedded ccording to the Falck-Hillarp procedure For Vibratome sectioning optimum results were obtained after perfusion with 150-300 ml ice-cold solution containing 4 % FA followed by immersion of the sections for 1 2 min in an ice-cold sol tion containing 5 g $MgSO_4 \times 7H_2O$ per 150 ml The sections were finally reacted with FA vapour For cryostat sectioning tissue perfused with the bove

FA-GA-$MgSO_4$ solution was used and the sections were immersed for 30 sec in an ice-cold 2 % GA solution before drying and FA vapour treatment (for details see Lorén et al 1976 1977)

The capability of the different $MgSO_4$ procedures to visualize CA and IA-systems was evaluated in the rat brai With respect to the CA-containing neuron systems ll techniques visualized the cell-bodies and their dendrites with high intensity as well as both terminal and non-terminal portions of the axons (Fig 1 a-d) Even the DA-containing terminals in th frontal cortex (which are possibl to demonstrate only with the most sensitive methods) were demonstrated with any of th techniques (Fig 1) However the DA terminals i the anterior cingulate cortex and the terminals of the incerto-hypothalamic DA ystem could be detected only with the modified freeze-drying and paraffin embedded procedure

The serotonin ystems were studied in rats pretreated with MAO-inhibitor lone or i combination with L-tryptophan Positive results were obtained only in the FA-GA-$MgSO_4$ perfused tissue processed for freeze-drying and paraffin embedding In such specimens the serotonin-contai ing cell bodies with their dendrites and proximal axons (Fig 2) were demonstrated with higher intensity and sharpness than in imilar specimens processed ccording to the standard Falck-Hillarp method (which currently is the most useful method for serotoni isualization) The serotonin-containing terminal systems were well isible in some areas such as i neocortex hippocampus globus pallidus substantia nigra and nc interpeduncularis

Of the different $MgSO_4$ procedures tested the freeze-drying paraffin-embedding method and the FA-V'bratome method gave the overall most satisfactory results whereas the cryostat procedure was somewhat less sensitive and less reproducible in our hands The present $MgSO_4$ modifications of the freeze-drying and FA Vibratome methods have sensitivity for NA and DA containing structures that is comparable to that of the GA-Vibratome method The $MgSO_4$-cryostat procedure although less sensitive than the alternative methods has in our laboratory given the best results of all available cryostat procedures on CNS tissue

The beneficial effects of high $MgSO_4$ concentrations could be explained by several factors It is highly probabl that the magnesium ions act to inhibit release of amines during processing and that the hypertonic salt solution help to dehydrate the tissue From preliminary model experiments it seems however that the most important factor is a direct action of magnesium ions on the fluorophore formation from the monoamines possibly by acting as Lewis acid in the catalysis of the histochemical reaction

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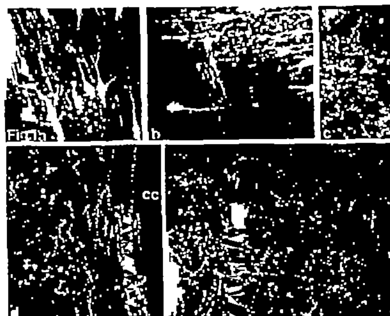


Fig 1 CA-containing cell bodies bundles and terminals sagittal sections a–c are from freeze-dried specimens d and are from Vibratome sections _ and b NA-containing cell bodies from nc subcoeruleus (X170); c: locus coeruleus terminals in the cerebellum (X 170); d part of the cingulum bundl just dorsal to the corpus callosum (cc) (X 190); _ DA and NA terminals i anteromedial frontal cortex (X 190)

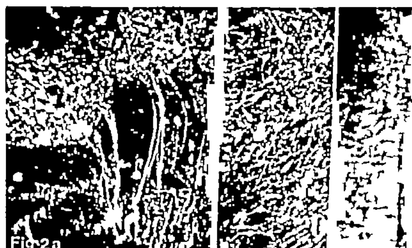


Fig 2 Serotonin–containing cell bodies axons and terminals from freeze–dried specimens sagittal sections _ cell bodies and proximal axons of neurons in the dorsal raphe nucleus (X 150); b: the ventral part of the median raphe nucleus together with serotoni terminals (X 170); c: serotonin–contai ing cell bodies and terminals underneath the ependyma of the IVth ventricle (arrows) (X 170) The animal was pretreated with chloral hydrate (300 mg/kg) talamide (300 mg/kg) and L–tryptophan (100 mg/kg)

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DISTRIBUTION OF ^{14}C-5 6-DIHYDROXYTRYPTAMINE IN THE CNS OF THE RAT

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5 6-Dihydroxytryptamine (5 6-DHT) has repeatedly been shown to reduce cerebral serotonin (5-HT) content when introduced into the CSF (1 2) The extent and time-course of long-term 5-HT depletion accomplished by 5 6-DHT wa found to vary with the region explored and not to be correlated with the dose of 5 6-DHT administered in certain CNS regions (1) In addition there is biochemical and morphological evidence for unselective damaging effects of 5 6-DHT on central NA (noradrenaline) and DA (dopamine) systems (2 3) These findings have been explained by physicochemical properties of the drug (its lability and difficulty to penetrate brain matter) and by peculiarities of the anatomy of the central 5-HT system (which is still imperfectly known at present) However it remained unexplained why 5 6-DHT caused toxic damage to some periventricular DA but not NA fibres since there is no difference in the affinity of 5 6-DHT to the central NA and DA uptak sites (2)
In order to answer th questions discussed above ^{14}C 5 6-DHT * (1-^{14}C 5 6-dihydroxytryptamine creatininesulfate H_2O 3 14 mCi/mmol) was injected into the left lateral ventricle of ther anaesthetized rats and the animals killed at 1 24 or 48 h after drug injection by intracardiac perfusion with saline containing

* ^{14}C-5 6-DHT was synthesized and donated by Dr H G Schlossberger Max Planck-Institute for Biochemistry Munich

10 % neutralized formol The brains were embedded in paraffin. Seril sections (5 μ) were coated with Ilford L 4 emulsion (dipping technique) and exposed for 6 weeks at 10°C The silver grains were developed with Kodak D 19 b and the sections counterstained according to Einarson

One h after ^{14}C-5 6-DHT silver grains are mainly found in a narrow zone of brain parenchyma (400–700 μ) bordering on the lateral IIIrd and IVth ventricle and on the basal cisternes of the subarachnoid space The number of grains in ventricle- and surface-near structures correlates with the known regional density and pattern of distribution of DA and 5-HT terminals and perikarya in the rat CNS Forebrain NA terminal systems and brainstem NA cells are much less intensely labeled The supraependymal 5-HT fibre plexuses are distinctly outlined (Fig 1c) in the ^{14}C-5 6-DHT injected brains Ventricle-near portions of the left caudate (Fig 1a) dorsolateral septum n accumbens septi (Fig 1a) olfactory tubercle (Fig 1b) and hippocampus have much more radioactivity incorporated than their contralateral homologues A decrease in the intensity of labeling of perikarya belonging to the various brainstem raphe nuclei is noted by 1 and 2 days but at the same time, an increased number of grains is found superimposed to certain monoaminergic fibre tracts (e g the median forebrain bundle and cingulum) Simultaneously scattered grains are noted over some surface- and ventricle-distant structures (e g the deep layers of the colliculi) By 24 and 48 h increasing numbers of radioactivity loaden macrophage-like cells are seen to accumulate in the ventricle- and surface near brain parenchyma on top of the ependymal cells and in the pia-arachnoidea



Fig.1 a,b,c:Autoradiograms of the n caudatus and accumbens septi (a) olfactory tubercle (b) and habenular nucleus (c) following intraventricular injection of ^{14}C-5 6-DHT

The present findings demonstrate that the penetration of 5 6-DHT from the ventricle into the brain parenchyma is in fact rather limited and that 5 6-DHT reaches only those surface-bordering regions that have access to the cisternes of the subarachnoid space The absence of toxic effects of 5 6-DHT on central NA neurons can be explained by a comparatively inefficient accumulation of 5 6-DHT in NA fibres and cells This finding suggests that the affinity of 5 6-DHT to the NA uptake sites in brain should be lower than to the DA uptake sites in discrepancy with earlier conclusions based on published data concerning the inhibition of the uptake of ^{3}H-DA and ^{3}H-NA by 5 6-DHT (4) Measurements of the apparent affinity of labeled 5 6-DHT to the NA and DA uptake sites will be required to solve this problem The time-dependent changes in the extent of labeling of perikarya and non-terminal monoaminergic axons are interpreted as indicating an intraneuronal dislocation of radioactivity by axoplasmic transport Finally the penetration of 5 6-DHT into the brain parenchymy and the selectivity of its accumulation in 5-HT NA and DA systems can be modified by drugs known to act on CNS monoamine mechanisms such as desmethylimipramine benztropin and monoamine oxidase inhibitors

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ON THE RELATION BETWEEN CENTRAL NORADRENALINE AND SEROTONIN NERVE TERMINALS AND POSTSYNAPTIC RECEPTORS DURING ONTOGENY

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Previous studies have shown that central noradrenaline (NA) and 5-hydroxytryptamine (5-HT) neurons undergo marked changes in their postnatal development following a neonatal administration of the neurotoxins 6-hydroxydopamine (6-OH-DA) and 5 7 dihydroxytryptamine (6-HT) respectively (1-6) Treatment with these neuroto ins at birth thus causes marked and permanent NA and 5-HT denervations in distant projections g in the cerebral cortex whereas in cell body near projections in the pons and medulla oblongata there is an increased number of nerve terminals This rearrangement of the NA and 6-HT nerve terminals have been interpreted to be due to mainly a "pruning effect (7)

Since the neonatal neurotoxin treatment leads to pronounced changes in the ontogeny of NA and 5-HT nerve terminals where the cerebral cortex and the pons-medulla represent the extreme regions(marked denervation and hyperinnervation respectively) it was thought of interest to study the accumulation of C AMP (adenosine 3 5 -monophosphate) elicited by NA and ^{3}H 5-HT receptor binding in these regions in the adult stage after a neurotoxi treatment at birth with iew to obtain some information as to the relationship between post synaptic recepto ontogeny and differentiation and maturation of NA and 5-HT nerve terminals

The basal formation and accumulation of C AMP was simila in 6-OH-DA treated and controls in both regions studied (Fig 1) There was however a marked increase in C AMP formation elicited by NA in the neocortex after 6-OH-DA treat ment at birth which can be interpreted as a supersensitivity response following the 6-OH-DA induced NA denervation in this region I the NA hyper-

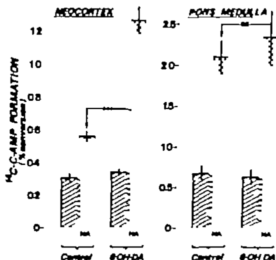


Fig 1 Effect of neonatal 6-OH-DA treatment (2x100 mg/kg s c) on the accumulation of ^{14}C C AMP in slices from the neocortex and pons-medulla pre-incubated with ^{14}C adenine (14 µM) and thereafter incubated with L NA (100 µM) according to the method of Shimizu et al (8) Each column represents the mean + S E M of 5-6 determinations, expressed as per cent conversion of total radioactivity in the slices to ^{14}C C AMP *** p<0 001; ns not significant

innervated region the pons-medulla there was no difference in C AMP formation

The studies on the in vitro receptor binding of ^{3}H-5-HT showed a saturable ^{3}H 5-HT binding in both regions investigated (Fig 2) It was consistently found that the 5 7-HT treatment at birth did not alter the binding characteristics and the maximal number of ^{3}H-5-HT binding sites neither in the cerebral cortex nor in the mesencephalon-pons-medulla (Fig 3)

The present results from the cerebral cortex both with respect to C AMP formation and ^{3}H-5-HT receptor binding indicate that the postsynaptic receptors develop independent of the presynaptic nerve terminals although it can not be excluded that NA or 5-HT present in the cerebral cortex before the neurotoxin treatment has triggered receptor formation during the postnatal development The observation in the mesencephalon-pons-medulla that ^{3}H-5-HT receptor binding is unchanged afte 5 7-HT treatment are also consistent with the view that the postsynaptic recepto ontogeny is independent of the presynaptic structure

It has been reported that there is an increased affinity for ^{3}H-5-HT binding

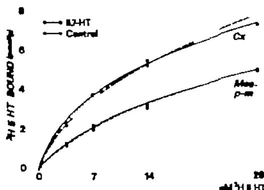
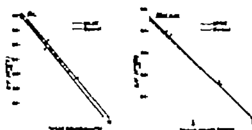


Fig 2 Effect of neonatal 5 7 HT treatment (100 mg/kg s c) on the in vitro binding of ^{3}H-5-HT to homogenates from the cerebral cortex (cx) and mesencephalon-pons-medulla (mes p-m) according to the method of Bennett and Snyder (9) The homogenates were incubated with various conc of ^{3}H-5-HT (3 5 28 nM) in the presence (non-specific binding) and absence (total binding) of 10 µM unlabelled 5-HT The values presented were obtained by subtracting non-specific binding from total ^{3}H-5-HT binding Each point represents the mean of 6-9 determinations

without any change of number of binding sites in the forebrain afte 5-HT denervation in adult rats (9) Such a change was not seen afte neonatal 5 7-5-HT treatment in the cerebral cortex in the present study The reason for this might be related to that 5-HT denervation (50-60% decrease) was not as marked as in the study ref rred to above As to catecholamine receptors it has recently been reported that a 6-OH-DA induced NA denervation in the cerebral cortex i adult rats is associated with an increased number of β-receptors (10)



F g 3 The ^{3}H-5-HT binding data from Fig 2 presented in a Scatchard plot for determination of equilibrium dissociation constants (K_D) and maximal numbe of binding sites (B_{max}) Cx K_D 16 nM and B_{max} 12 pmol/g Mes p-m: K_D 15 nM and B_{max} 7 pmol/g

Recent experiments in this lab have also shown that a neonatal 6-OH-DA treatment leads to an increased number of β receptor binding sites in the neocortex (Jonsson unpubl data) Therefore it seems as if a denervation may for certain systems (5-HT ref 9) lead to a change in the affinity of the transmitter to the receptor while for other systems (NA) to an increase in the density of the receptor sites Both these changes may be of importance for a supersensitivity response following a denervation

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SPECIES DIFFERENCES IN SEROTONINERGIC INNERVATION AND SECRETORY ACTIVITY OF RAT GERBIL MOUSE AND RABBIT SUBCOMMISSURAL ORGAN

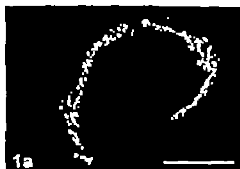
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The subcommissural organ (SCO) is specialized area of the ependyma attached to the ventral surface of the posterior commissure where it forms the lining of the roof of the cerebral aqueduct at its junction with the third ventricl The function of the SCO remains obscure but the pronounced secretory cti ity of the organ is well established Secretory material of glycoprotei character is released apically into the ventricular fluid and forms the so-called Reissner fibre In addition basal secretion i to the blood vessels is postulated in some species (cf 1)

We have recently reported that the SCO of the adult rat receives dense indolaminergic (probably serotoninergic) synapti innervation which seems to inhibit both the syntheti and secretory activities of the specific SCO ells (2) The SCO of the neonatal rat is not innervated but it is not known how the establishment of serotoninergi innervation during the second postnatal week (3) affects the secretory activity of the organ In the present study we have investigated if serotoninergi innervation of the SCO exists i number of rodent species and related this to the secretory activity of the organ i neonatal and adult animals

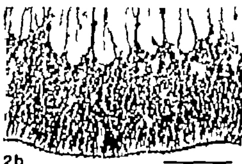
The material comprises neonatal and adult rats mongolian gerbils mice and rabbits The animals used for fluorescence histochemistry were pharmacologically pretreated to increase intraneuronal serotonin content (cf 4 5) and then processed for fluorescence histochemical detection of monoamines according to Falck and Hillarp (for technical details see 6 7) Brains used for histochemical detection of secretory material were rapidly removed after decapitation and fixed for 24 hours in formol cal ium or for 72 hours in Bouin fixative (8) Serial sections of 3 microns were cut through the entire organ of each animal The sections were stained with either toluidine blue periodi acid-Schiff following previous digestion by maltase or by Bock chromalum gallocyanin (9)

Fluorescence microscopically dense uniform plexus of brightly yellow fluorescent nerve fibres is observed in relation to the basal part of the entire SCO of the rat (Fig 1) The basal part of the gerbil SCO lso receives an indolaminergic input (Fig 1 b) but the













Figs 1 b and c Note the fluorescent innervation (serotoninergic) of the adult rat (a) and gerbil (b) SCO The innervation is more dense and of higher fluorescenc intensity in the rat SCO compared to that of the gerbil while innervation is absent i the rabbit SCO (c) Figs 2 b and demonstrate the differences in the distribution of Gomori-positive material Virtually no reaction is seen in the SCO of the adult rat (2 a frontal section) while the neonatal gerbil (2 b) and the adult rabbit (2 c) exhibit very strong reaction (sagittal sections) Bars on all figures indicate 100 microns

innervation is more sparse and the nerve fibres exhibit weaker fluorescence than i th rat In marked contrasts to the observations in rat and gerbil we found no evidence of sero- toninergi innervation of the mouse (not shown) or the rabbit SCO (Fig 1 c) Neonatally none of the investigated species including rat and gerbil, show indolaminergic innervation of the SCO

In general two sorts of Gomori-positive material can be distinguished histochemically in SCO cells; fine granular substance is found at the level of the nucl i and some strongly reacting large granules accumulate ither in the basal processes close to the blood vessels or in the apical processes adjacent to the ventricular surface A very low reactivity is found in both apical and basal processes of the SCO of adult rat (Fig 2) and gerbil as opposed to the strong activity found in mouse and rabbit (Fig 2) In these two species an intensively stained fine granular substance seems to load most cells and i addition strongly reacting granular material is found in both apical and basal processes The SCO of neonatal rats and gerbils (Fig 2 b) exhibits strong reactivity nearly imilar to that found i SCO of adult mouse and rabbit, whereas the organ has lower reactivity i neonatal mouse and rabbit

The findings of this study indicate an inverse relationship between serotoninergi inner- vation and secretory activity in the SCO Thus in animals (rat and gerbil) which post- natally develop serotoninergi innervation of the SCO the secretory activity of the organ will be markedly decreased in th adult These observations are i line with the results of our experiments with chemically induced denervation of the rat SCO (2) which indicate that the serotoninergi innervation exerts powerful inhibition of secretory and synthetic activity of the SCO We have found that the SCO provides an important model for the study of serotoninergic function

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OLFACTORY BULB DOPAMINE NEURONS - THE A15 CATECHOLAMINE CELL GROUP

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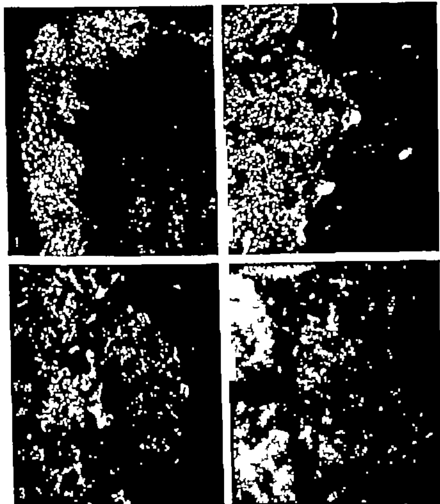
On the basi of the F lck Hillarp formaldehyd fluorescence meth od (1) 14 c techolamine containi g cell groups have been id nti fied in the rat b ain (2) More recently immunocytochemistry h s been employed in tudi on monoamin neurons Geff n and collaborators (3) used an anti rum to dopamine-β hydroxylase (DBH) to ide tify catecholamine neurons in th peripheral nervo system Subsequently several groups h ve applied the same principal approach using not only antibodie to DBH but also to other enzyme in the techolamine synth i In the present communi tion we focus the ttention on the exi tence of small neurons in the lf ctory bulb which contain tyrosine hydroxylas (TH) and DOPA-decarboxylase (DDC) but not DBH Thi trongly suggests that the olfactory bulb contains dopamine neurons

Mal albino ts were perfused with ice-cold formalin and th ol factory bulbs wer di ected out immersed in th ame fixative rinsed out on a cryostat and processed for the indirect immunofluorescence technique of Coons and collabor to (see 4) Bri f ly se ie of sections wer incubated with anti erum to TH DDC DBH co trol serum After in ing the secti ns were incubated

with fluoresceinisothiocyanate (FITC) conjugated sheep anti rabbit antibodies rin ed mounted and examined in fluorescence mi ro-scope For furth r details see 5

After incubation with antiserum to TH (Figs 1-3) and DOC numerous fluorescent cell bodies were observed in th glomerular la y r of the olfactory bulb The cell bodies we e localized in the periphery of glomeruli The interio of the glomeruli cont i ed numerous fluoresce t fibers sometimes in continuity with the cell bodies There was an impression of more DOC than TH immunoreactive fibers Singl fluorescent cell bodies could also be observed in the external plexiform layer after incubation with th se two nti ser I addition weakly TH positive fibers were observed i th external plexiform nd g nul r l yers sometimes also in the glomerular layer After incubation with DBH antiserum (Fig 4) no immunoreactive cell bodies were seen but a moderately dense network of fluorescent fibers wa ob rved in the external plexiform and granular layers and occasionally in the glomerular layer

The pre ent findings give strong evidence for the view th t a certain population of so called periglomerular cells (see 6) synthesize and store dopamine extending early studies by Dahlström _et_ _l._ (7) and Lichtensteiger (8) with the formaldehyde fluorescence method indicating the existence of catecholamine neurons in the ol factory bulb Since 14 catecholamine cell groups have been d scri bed so far we suggest that the intrabulbar periglomerular dopamine cells are assigned as the A15 group according to the nomenclature of Dahlström and Fuxe (2) The presence of dopamine synthesizing enzymes in the dendrite of the periglomerul r cells as well a



Figs 1 4 Immunofluorescence mi rogr ph of the at olfactory bulb after incubation with TH anti erum (Fig 1 3) or DBH nti e-rum (Fig 4) Numerous TH-po itive ells and process seen in the glomerul layer wherea only a few DBH positive nerve termi-nal ar pr sent (Fig 4) Figs 3 and 4 show consecutive sections Magnifications 100X (Fig 1) and 250X (Fig 2 4)

the known d ndro-dendritic synapses formed by this type of neurons (see 6) suggest that dopamine may be released from dendrites in the glomeruli

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SUBCORTICAL AFFERENTS TO THE PREFRONTAL CORTEX ORGANIZATION OF MESENCEPHALIC DOPAMINERGIC AND MEDIODORSAL THALAMIC PROJECTIONS

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It is now generally agreed that the best definition of th prefrontal cortex (PFC) is the neocortical projection field of the mediodorsal thalami nucleus (MD; 1 2) Experiments with cortical ablations have indicated that the PFC plays imilar functional roles i brains of widely different species (see ref 3) In series of studies (4 5 6 7) we have used the horseradish peroxidase (HRP) technique i combination with fluorescence histochemistry and various types of lesions to describe the subcortical input to PFC The following gives brief review of our findings

I agreement with previous studies (8 9 10) MD was found to project densely to the anteromedial cortex and the dorsal bank and lip of the anterior third of the rhinal sulcus in the rat thus defining the PFC i this species MD was lso shown to project sparsely to the dorso-polar frontal cortex HRP i jections into the subfields of PFC labelled perikarya i different divisions of MD The injections i the ventral pregenual part of the medial surface labelled predominantly th dorsomedial part of MD whereas more dorsal placements resulted in labelling of the cells i the lateral and ventral parts The supragenual i jections labelled the cell bodies i the postero-dorso-lateral region of MD and the injections in the dorsal bank of the anterior portion of the rhinal sulcus produced labelling in the ventromedial part of the nucleus

With the HRP technique the cortical projection area of MD was found to coincide closely with that of the dopaminergi neurons in the ventral mesencephali tegmentum (4 5 9 11) The organization of the dopaminergi projections to PFC in the rat was investigated i more detail On basis of fibre morphology and distribution of fibres and cell bodies of origin three different dopaminergic terminal systems have been distinguished (Fig 1) The anteromedial system is formed by smooth axons originating i the medial part of the A10 cell group in the mesencephalon The axons are distributed mainly in the pregenual part of the anteromedial cortex and the highest fibre density is found in the basal cortical layers A much more sparse caudal extension of this system is present in the supragenual part of the anteromedial cortex Th suprarhinal system forms the dorsal part of the dopaminergi innervation of the perirhinal cortex surrounding the rhinal sulcus It can be

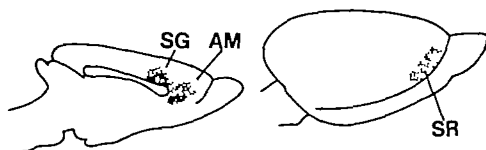


Fig 1 Schematic representation of the distribution of dopamine-containing terminals i the prefrontal cortex AM = anteromedial system; SG = supragenual system; SR = suprarhinal system

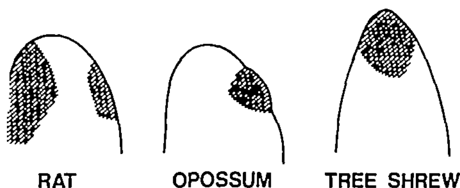


Fig 2 Position of the dopaminergic innervation and the MD projection fields in the frontal lobe of rat opossum and tree-shrew

regarded as direct lateral continuation of the anteromedial system and can be followed from coronal level just rostral to the nucleus accumbens to the level of the rostral part of th caudate-putamen The axons are distributed mainly in the basal cortical layers and have their cell bodies of origin in the dorsolateral part of the A10 cell group The supra genual system is formed by very fine-varicose axons distributed i a restricted area of the supragenual anteromedi l cortex. Th axons are localized in the superficial cortical layers (I III) and the cell bodies of origi are distributed in the ventrolateral A10 and long the mediolateral extent of the substantia nigra (A9)

W have lso tried to clarify whether the overlapping projections from MD and mesencephali dopaminergic cell groups in the rat is accidental or perhaps an exceptional feature of this species or whether there is consistent convergence of the two systems in different mammals For this purpose the afferent connections of PFC were studied in two species from different orders the opossum and tree shrew selected because they are presumed to resembl ancestors of mammals and primates respectively (12 13 14) In comparison with the rat the MD of the tree shrew and opossum projects to topographically different areas of the frontal lobe the frontopolar and dorsolateral frontal cortex respectively (Fig 2) However disregarding the different positions of PFC in the frontal lobe the area innervated by MD lso receives dense presumed dopaminergi projections from the ventral mesencephali tegmentum both in opossum and tree shrew By HRP i jections and fluorescence histochemistry the distribution of dopaminergi terminals f mesencephalic origi was shown to conform very well with the MD projection area

Our studies have shown that in three mammalian species the neocortical projection field of the mesencephalic dopaminergic neurons overlap lmost ideally with that of MD Interestingly Krettek and Price (15) have shown that lso the projection from amygdal converges with that of MD in the rat In the rat the dopaminergic innervation could be subdivided into three terminal systems with different origins and distribution Each of these systems converged with the projections from subgroup of cells in MD The noradrenergi and serotoninergi cell groups are known to have disperse projection patterns i the neocortex i they innervate diffusely ll neocortical areas (16 17) In contrast the present studies have demonstrated that the mesencephali dopamine cell groups have regional projection pattern in the neocortex and the dopaminergic innervation highly specific organization

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DEVELOPMENT OF DOPAMINE SENSITIVE ADENYLATE CYCLASE IN HIPPOCAMPUS REINNERVATED BY TRANSPLANTED DOPAMINE NEURONS EVIDENCE FOR NEW FUNCTIONAL CONTACTS

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The rat hippocampus is normally lacking any significant dopaminergi innervation However following grafting of embryoni mesencephali tissue to cavity made in the entorhinal cortex the dopamine (DA)–containing neurons in the transplant will sprout into the partly denervated hippocampus to form an anomalous DA–containing terminal network especially in the molecular layer of the dentate gyrus (1) I terestingly the patterning of the newly-established DA plexus conforms to the terminal fields of the temporo-ammonic perforant path fibres removed by the transplantation lesion Since these latter fibres are non-aminergic, possibly utilizing an excitatory amino acid such as glutamate as transmitter (2) these observations suggest that the ingrowing DA neurons might fill the vacated terminal space of non-aminergi ystem

In the present communication we report some initial findings from continuing series of experiments aimed at testing whether I the experimentally reinnervated hippocampus the anomalous DA terminal supply would form functionally normal connections despite the lack of dopaminergic synapses in the normal hippocampus The results show that concomitant with the rise i DA levels i the hippocampus DA sensitive adenylate cyclase appears possibly indicative of development of DA receptors in the reinnervated tissue

The region containing the mesencephali DA neuron system was taken from the brain stem of rat embryos (crown–rump length 15–25 mm) The tissue pieces were transplanted into the occipital pol of the cerebral hemisphere of adult femal Sprague–Dawley rats as described previously (3) All recipient rats were prior to transplantation subjected to bilateral intracranial sympathectomy and were given an intraventricular injection of 6-hydroxydopamine (250 µg free base) in order to remove the normal noradrenergi innervation of the hippocampus In the transplanted rats the hippocampus contralateral to the transplant served as control ide Additional control animals were subjected to ll the operational procedures but did not receive transplants All animals were killed 6–10 weeks after operation

In order to obtai some quantitative information about the extent and amount of DA ingrowth into the dentate gyrus and hippocampus samples were taken from the hippocampus

near the site of transplant ingrowth These samples were assayed for DA or dihydroxy-phenylacetic acid (DOPAC) by the use of COMT based enzymatic procedures (4) The tissue content of DA and DOPAC varied widely i particular in terms of the DA content of the re-innervated hippocampus (Table 1) The DA values were in range between 0 1-2 0 µg/gram presumably reflecting variations i extent of reinnervation survival of the transplants and position of the sampl taken for assay i relation to the ingrowth site The average content of DA 0 81 µg/gram (see Tabl 1) is in the range found for the septum or the central nucleus of the amygdala (5) and reflects a moderate dopaminergic innervation This concentration is however considerably less than in DA-rich areas such as nucleus accumbens neostriatum or olfactory tubercl

The levels of DOPAC i the hippocampus were considerably lower (Tabl 1) and were more consistent with areas such as frontal cortex having only minor DA innervation (6) As the DOPAC determinations were carried out on separate series of rats it is not known if these samples were just poorly innervated or whether the low level of DOPAC were due to low amounts of DA being released from the terminals

Table 1

Dopamine and DOPAC levels in reinnervated and normal hippocampus Means ± S E.M Number of determinations i brackets * = $p < 0\ 05$ (t-test)

	Normal hippocampus (µg/gram)	Reinnervated hippocampus (µg/gram)
Dopamine	0 025 ± 0 004 (6)	0 81 ± 0 36 (8)
DOPAC	0 022 ± 0 003 (8)	0 046 ± 0 006 (8)

All tissues so far studied which are known to receive DA inputs contain DA sensitive adenylate cyclase which is believed to be linked to the postsynaptic DA receptor (7) It was thus of great interest to see if the ingrowth of DA terminals into the hippocampus resulted in the appearance of a DA sensitive adenylate cyclase within the reinnervated tissue In many cases addition of DA (100 µM) to homogenates of the reinnervated hippocampi resulted in stimulation of the enzyme activity which could be blocked by α-flupenthixol (10^{-6}M) (Tabl II A and B) The maximum stimulation was about 100 % above basal value with an average of 45 % This response like the DA content of the hippocampus was

Table II

Dopamine-sensitive adenylate cyclase i reinnervated and control hippocampus and in striatum.) Means + S E M. Number of determinations in brackets * = $p < 0\ 05$ (paired t-test)

A Cyclic AMP production (pmoles/2.5 min/mg protein)

	Basal	+ DA (10^{-4} M)	+ DA (10^{-4} M) + + α-flupenthixol (10^{-6} M)
Control side	67 3 ± 5 3 (16)	72 5 + 8 3 (16)	
Transplant side			
exp 1	66 6 ± 7.2 (8)	96 6 + 12.2 (8)	
exp 2	79.5 + 5.7 (6)	92.2 ± 9 4 (6)	77 7 ± 2 6 (6)
Striatum	122 0 + 6 6 (8)	204 0 ± 15 3 (8)	125 0 + 1 6 (4)

B Range of adenylate cyclase activity in 14 *reinnervated hippocampi (units as in A)

Rat No	Basal	+ DA (10^{-4} M)	Rat No	Basal	+ DA (10^{-4} M)
1	66 0	86 4	8	93 0	174 0
2	81 0	96 0	9	57.5	59.5
3	52.5	49.5	10	96 0	126 0
4	58.5	84 0	11	88.5	88.5
5	24 0	36 0	12	90 0	90 0
6	55.5	54 0	13	63 0	96 0
7	61.5	72 0	14	57 0	99 0

) Adenylate cyclase activity was determined with the method of Kebabian et al (8)

variabl and was not seen in several animals (Table II B) nor in control animals with entorhinal lesion lone The absence of this effect in lesioned controls indicates that it is not due to a onspecific response of the tissue to the operative procedures Although the response is to DA and is blocked by α-flupenthixol, dose-response curves and responses to β-blockers and β-receptor stimulants are being carried out to further characterize the receptor involved However the occurrence of this response in homogenates and the blockade of the response by α-flupenthixol makes it likely that we are dealing with a DA receptor linked adenylate cyclase (see ref 7)

Current evidenc is consistent with the belief that the DA sensitive adenylate cyclase is linked to the postsynaptic DA receptor (7) If this is the case lso in the experimentally reinnervated hippocampus then this may be the first example of an actual induction f a new postsynaptic receptor after reinnervation of normally non-dopaminergi terminal area Experiments are now i progress to investigate the location of this DA sensitive adenylate cyclase

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THE ORIGIN OF SUBSTANCE P AND ACETYLCHOLINE PROJECTIONS TO THE VENTRAL TEGMENTAL AREA AND INTERPEDUNCULAR NUCLEUS IN THE RAT

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In 1931 Von Euler and Gaddum reported the existence of vasoactive peptide substance P in the brain and gut (1) The isolation and synthesis of substance P by Leeman and her colleagues (2 3) has allowed the application of sensitive radio-immunoassay and immuno-histochemical techniques to the study of the distribution of substance P in the central nervous system (CNS) Substance P like material i concentrated in specific regions of the rat CNS where it is localized in neuronal cell bodies and terminals from which substance P like material can be released by calcium-dependent mechanism (4) More recently vesicular localization of substance P has been demonstrated (5 6) These observations are consistent with the view that substance P is neurotransmitter i number of neuroanatomical pathways (7)

In earlier work i parallel with other groups we reported the existence of habenular-interpeduncular pathway containing both substance P like material and choline acetyltransferase (ChAT) cholinergi marker enzyme (8) I this report we describe further work which allowed us to dissociate the cholinergi and substance P projections to the interpeduncular area

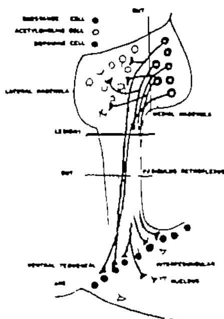
Mal Sprague-Dawley rats (300 g) were subjected to micro-knif lesions designed to (a) separate the medial and lateral habenular nuclei unilaterally or (b) to sever the *fasciculus retroflexus unilaterally Animals were sacrificed at different times after these* lesions and the following nuclei dissected: ipsi- and contralateral medial and lateral habenular nuclei fasciculus retroflexus above the transection and the ventral tegmental area-interpeduncular nucleus region Samples were homogenised and extracted for assay of substance P like material (9) or for assay of ChAT (10)

The results of experiments designed to separate the medial habenular nucleus from the lateral habenular nucleus are shown in Table 1 This lesion (cut 1 Figure 1) which isolates the medial nucleus from the lateral nucleus and transects the efferent pathway from the medial nucleus long the fasciculus retroflexus depleted the medial nucleus of its content of ChAT but not of substance P The lateral nucleus which retains its projection to i terpeduncular area was depleted of substance P but not of ChAT In the interpeduncular area

Tabl 1 ChAT activity and substance P levels I the habenula-ventral tegmental pathway after selective lesions as shown in Fig 1 Each value is the mean of at least 4 determinations

	REGION	ChAT % of control	Substance P % of control
Lesion 1	Ventral tegmental area	9.5	28 6
	Medial habenula	23 1	105 2
Cut 1	Lateral habenula	107 6	57 1
	Ventral tegmental area	116 2	48 0
	Habenula	113.2	109 9
Cut 2	Fasciculus retroflexus	189 9	225 0
	Ventral tegmental area	41 0	54 7

Figure 1 Projection of Substance P and acetyl choline containing neurons from the habenula to the ventral tegmental-interpeduncular area



significant depletion of substance P was observed but not of ChAT Experiments transecting the fasciculus retroflexus (cut 2 Figure 1) however depleted the interpeduncular area approximately equally f its substance P and ChAT content and produced an accumulation of substance P and ChAT above the knife cut (Tabl 1) (10)

These results taken together with parallel histochemical studies of AChE and substance P distribution (11) indicate that the medial habenular nucleus contains substance P neurons which project via the fasciculus retroflexus to the ventral tegmental area (see Figure 1) These neurons also send substance P containing axons or collaterals to the lateral habenular nucleus The substance P projection from the medial habenular nucleus is mainly to the ipsi lateral ventral tegmental area where it corresponds to the organisation f the dendrites of the dopamine cells i this area (11) an interesting parallel with the substantia nigra where the striato-nigral substance P projection also parallels the distribution of DA dendrites i the substantia igra The lateral habenular nucleus sends cholinergi efferents via the fasciculus retroflexus which terminate evenly throughout the i terpeduncular nucleus and axons or collaterals which lso innervate the medial habenular nucleus Our proposed scheme of organisation of the habenula–ventral tegmental area–interpeduncular nucleus projections is illustrated in Figure 1 The ability to dissociate substance P and cholinergi projections to the interpeduncular area indicates that the suggestion made by Mroz et al (13) that both substance P and acetylcholine might be i th same neurons in the habenula is incorrect and that in the rat CNS at least Dal law (14) is still intact

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DESTRUCTION OF AMINE ACCUMULATING NEURONS IN RABBIT AND GOLDFISH RETINA

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When the fluorescence microscopical method of Falck and Hillarp was i troduced it was soon discovered that there was a previously unsuspected set of dopamine containing neurons in the rat retina (1 2) and continued studies have shown them to occur (with some variations) in all vertebrates (3) Recently a system of indoleamine accumulating neurons has also been discovered (4 5) The transmitter of these neurons is not accurately known but is most likely an indoleamine perhaps serotonin In any event these neurons are demonstrable i the fluorescence microscope only after intraocular injections of indoleamines They then show yellowish fluorescenc but the dopaminergic neurons also accumulate some of the indoleamine and therefore also get a yellowish colour Normally the dopaminergic fibres show greenish fluorescence but when they have accumulated an indoleamine they may be very difficult to distinguish precisely and in detail from the proper ndoleamine accumulating neurons particularly when the two types occur together in den e meshworks like they do in the inner plexiform layer Further the methods available for making aminergic neurons isible in the lectron microscope (6 7) does not distinguish between the two types There was therefore a need for procedures that can remove either the dopaminergic or the indoleamine accumul ting neurons Further ery l ttle is known about the f nction of either neuron type i the retina A classical approach for functional analysis is to remove the structure under investigation and to study the ensuing defects in the funct on Methods for removal of the aminergic neurons re thus of importance also from this point of view

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A CATECHOLAMINERGIC NEURON CONNECTING OPTIC NEUROPILES OF CRAYFISH

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Introduction The understanding of the visual integration processes in arthropods has been enhanced by the characterization of neuron types using the selective Golgi silver impregnation technique (1 2) With the histochemical fluorescence method of Falck Hillarp (3 4) the presence of specific and distinctly localized green fluorescent layers in the optic neuropiles of insects and crus taceans was demonstrated (5) With the introduction of exogenous catecholamines after reserpine treatment the neuronal uptake is cytoplasmic and the histochemical fluorescence method reveals the whole neuron Thus a comparison can be made between the results obtained on neuronal topography with the silver impregnation and fluorescence techniques The present investigation aided by ultrastructural analyses has attempted this and the results have revealed a difference in this case between the neuron types found with these two tech iques

Material and Methods The crayfish Pacifastacus leniusculus was used in the experiments

With repeated administration of 10-25 mg of reserpine (Serpasil[R] CIBA) per kg body weight on three consecutive days the fluorescence in the optic neuropiles disappeared On the fourth day the e cised optic neuropiles were incubated at room temperature in 0 1-0 2 µg/ml α-methylnoradrenaline or 1 2 µg/ml dopamine with 0 2 µg/ 1 ascorbic acid in crustacean saline (6) for 30 min After this treatment the normal procedure with freeze-drying paraformaldehyde vapour treatment and embedding in paraffin followed (for details see 4) For electron microscopy an aldehyde prefixative according to Karnovsky (7) was used (3 hours) Postfixation followed with 2 % OsO_4 for two hours Cacodylate buffer was used throughout The tissue was blockstained with 1 % phosphotungstic acid and 0 5 % uranyl acetate and finally embedded in Vestopal W

Results The two distal optic neuropiles in the eyestalk of Pacifastacus leniusculus the lamina ganglionaris the medulla externa (Fig 1) are of special concern i this investigation They are connected by diff rent kinds of neurons forming a chiasma (8) These neuron types are recently characterized in a Golgi tudy on the lamina by Nassel (2) Seven of the eight photoreceptor

axons terminate in two substrata (epl_1 and epl_2) of the first plexiform layer (lamina) The five types of monopolar neurons connecting the lamina with the medulla branch in one or both of these substrata Two types of lamina-medulla tangential neurons have been found and these are of special interest in this investigation Further multipolar and centrifugal neuron types have been described but these are not further considered here

In the histochemical fluorescence method of Falck-Hillarp a green fluorescent compound is formed which invariably appears as a broad distal band followed by two thinner bands proximally in the medulla externa Only rarely is a fluorescence found in part of the lamina and never in the chiasma

Administration of exogenous catecholamines after reserpine treatment makes the fluorescence picture more complete (Fig 1) The lamina ganglionaris the chiasma and the same layers of the medulla as mentioned above show an intense fluorescence which is also the case with a large number of cell bodies in the cell-body layer belonging to the medulla externa The arborizations of the catecholaminergic neuron type within the lamina consist of axons penetrating the whole thickness of the lamina and giving off lateral branches in two strata one in the distal and one in the proximal margin The termination in the medulla e terna constitutes the broad fluorescing distal layer of that optic neuropile Each fibre seems to have a restricted lampbrush like terminal extending the complete depth of the stratum (Fig 1)

Ultrastructural investigations of the normal lamina ganglionaris display fibre profiles containing dense vesicles Two types of fibres can be distinguished by their different vesicle content Type 1 has true dense core vesicles Type 2 is presumed to be neurosecretory The overall picture gained of the spatial arrangement of type 1 fibres shows an axon entering the lamina and splitting up into a proximal and a distal layer of branches It is obvious that this correlates well with the pattern seen in the fluorescence microscope



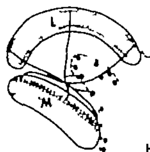


Fig 1 a) Horizontal section through the lamina ganglionaris (L) and the medulla externa (M) incubated in dopamine after reserpine treatment Only the distal thick fluorescent layer is seen in the somewhat obliquely sectioned medulla (scale 100 μm)

b) A schematic drawing of the same portion illustrating the catechol aminergic tangential neuron Dotted areas indicate green fluorescence hatched areas cell-body layers and open circles fluorescent cell bodies

Discussion The catecholaminergic neuron type characterized in the present investigation is identical with that which only partially fluoresces in the standard Falck-Hillarp method The spatial distribution of the fluorescence in the medulla and lamina along with the ultrastructural findings of normal lamina ganglionaris (type 1 fibres) supports this idea The catecholaminergic neuron type will be designated as the catecholaminergic tangential neuron (CTN) Two such types (Tan_1 and Tan_2) have been described previously from the lamina of Pacifastacus leniusculus (2) They differ from CTN and it is thus a new tangential T neuron type It is to be noted in this context that the CTN is not vi ualized in Golgi preparations Although catecholaminergic neurons may accept silver they are less likely to do so For the present the two techniques can be said to complement one anothe to a significant degree

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Section II

EXTRINSIC INNERVATION OF THE CEREBROVASCULAR BED

Acta physiol. scand. Suppl. 452, 1977

SYMPATHETIC INNERVATION AND ADRENERGIC RECEPTORS IN INTRAPARENCHYMAL CEREBRAL ARTERIOLES OF BABOON

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Ampl evidence has demonstrated that the pial arterial system of several mammalians including humans I innervated by autonomic nerves and that the vascular tissue possesses amine receptors (1) The present xperiments on baboons were undertaken to evaluate to which extent the intraparenchymal arterioles in various regions are innervated and have amine receptors

Several brain regions were dissected out and immediately frozen to the temperature of liquid itrogen The specimens were freeze-dried, treated in formaldehyde gas for 1 hr at 80 C sectioned at 6 µm thickness and processed for fluorescence microscopy (2)

The network of sympatheti adrenergi nerves enclosing the pial arteries accompanied several of the arterial branches as they issued into the brain parenchyma (Fig 1) and innervated arteriolar branches could be followed deep in the brain (Fig 1b) The pattern of innervation showed regional heterogeneity as demonstrated in Fig 2.

Intracerebral arterioles in the anterior part of the brain were dissected out and placed in ice-cold oxygenated Krebs-Ringer buffer solution Five mm long segments were mounted between two L-formed metal prongs for recording of circular vasomotor activity as previously described (3)



Fig 1 Fluorescence photomicrographs of sections from the cerebral corte of baboon showing (left X 225) penetration into the parenchyma of sympathetically innervated branch from pial artery and (right X 350) longitudinally sectioned segment of a parenchymal arteriole with well-developed adrenergic innervation running deep in the cortex

0 20 40 60 80 100 %

Area
Medial geniculate body
Inferior colliculus
Parietal cortex
Hypothalamus
Thalamus
Superior colliculus
Caudate nucleus
Temporal cortex
Periventricular zone
Spinal cord
Hippocampus
Frontal cortex
Lateral geniculate body
Olfactory bulb
Medulla oblongata
Occipital cortex
Cerebellum

0 20 40 60 80 100 %

Fig 2 Fluorescence microscopic estimation of the percentage of arterioles in various brain regions of baboons supplied by adrenergic sympathetl nerves

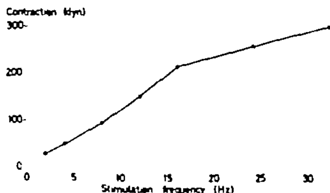


Fig 3 Transmural lectrical stimulation (12 V and 1 msec pulse duration) of the adrenergic nerves in pial artery from baboon producing frequency-dependent contractile response of the vessel

Electrical fi ld stimulation induced vessel contraction whose strength increased with increasing stimulation frequency (Fig 2) This effect could be antagonized by the presence of the specific alpha adrenergic blocking agent phentolamine in the organ bath The vascular segments were also contracted i dose dependent manner by noradrenaline the effect being inhibited i competitive manner by increasing doses of phentolamine Provided the vessels had been given an active tone isoprenaline i increasing doses dilated the arterioles This response was inhibited by the beta blocking agent propranolol

Con lusion: Intraparenchymal vessel f baboons are innervated by sympatheti adrenergi nerves There is regional heterogeneity in the extent of innervation probably related to varying regional flow response to sympatheti activation or sympathomimeti compounds The vascular ffects of sympathomimeti agents are mediated by constrictory alpha-receptors and dilating beta-receptors

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IMMUNOHISTOCHEMICAL DEMONSTRATION OF VASODILATORY PEPTIDERGIC NERVES IN BRAIN

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In the course of electron microscopic studies on the pial vascular innervation (1), nerve terminals were found whose content and structure of synaptic vesicles did not conform with either adrenergic or cholinergic nerves. Further attempts to identify peptidergic nerves in relation to brain vessels gave positive results with vasoactive intestinal peptide (VIP), which could be localized in cerebrovascular nerves by immunofluorescence. VIP is a potent hypotensive and vasodilatory agent, first isolated from porcine small intestine, and shown to have an amino acid sequence related to that of secretin, glucagon, and gastric inhibitory polypeptide.

Antisera to porcine VIP, covalently bound to bovine serum albumin, were raised together with Freunds complete adjuvant in rabbits. The antigen-antibody reaction was revealed with fluorescein isothiocyanate-labelled sheep anti-rabbit IgG. The VIP antiserum employed has previously been well characterized (2,3). No cross-reactivity with a number of polypeptide hormones, including somatostatin and substance P, was found. The antiserum was inactivated by the addition of porcine VIP. Seven different VIP antisera were tested at the immunohistochemical level, and all produced staining of the perivascular nerves.

Fibres containing VIP-immunoreactivity were seen in the adventitia or adventitia-media border of most of the pial vessels (Fig. 1). A moderate number of single fluorescent fibres, or small fibre bundles, ran long and close to the vertebral arteries. In the basilar artery the fibre branches extended more deeply into the vessel wall. Only very few immunoreactive fibres were associated with the superior cerebellar arteries. A much richer supply was found in the anterior portion of the circle of Willis. Thus, a very well-developed plexus of fluorescent fibres with beaded (varicosed) appearance enclosed the anterior cerebral artery. An almost equally dense plexus of VIP-immunoreactive fibres was found in the wall of the proximal portion of the middle cerebral arteries, posterior communicating arteries, and in the small arterial branches derived from this area. Occasionally immunoreactive fibres have also been observed along intracerebral vascular branches. Cell bodies with VIP-immunofluorescence were present in the superior cervical sympathetic ganglia.



Fig 1 VIP immunoreactivity in transversely sectioned anterior cerebral artery from cat The picture i composed of two adjacent sections and shows numerous delicate nerves (some indicated by arrows) enclosing the vessel in the adventitia media border Magnification 190 X

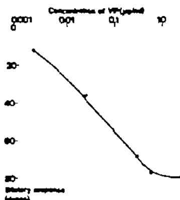


Fig 2 Dose-related dilatation of cat middle cerebral artery in vitro in the presence of VIP added in a cumulative way The vessel was given an initial active tension of 200 dynes by 3 10^{-6} M of serotonin

VIP produced strong dose-dependent vasodilatation of isolated cat s middl cerebral artery in vitro (Fig 2) in the same order of magnitude as that produced by acetylcholine or isoprenaline However the effect does not seem to be mediated by β-adrenergic or cholinergi muscarini receptors

Conclusion Immunohistochemistry has revealed hitherto unknown system of peptidergi cerebrovascular nerves with dilatory ffects

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INFLUENCE OF THE CEREBROVASCULAR SYMPATHETIC INNERVATION ON BLOOD-BRAIN BARRIER FUNCTION

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The brain vascular bed receives an ampl supply of sympatheti adrenergic nerves originating in the superior cervical ganglia (1) These nerves may at least during certain conditions modify the cerebral blood flow (2) There is evidence supporting the assumption that the nerves have physiological rol reflected in for example the recent observation that stimulation of the cerebral sympatheti nerves extends the upper limit of autoregulation towards higher blood pressure (3 4)

The influence of the cerebrovascular sympatheti nerves on another cerebrovascular function the blood-brain barrier was studied using Evans blue-albumin comple as marker during angiotensin-induced acute systemi hypertension Male Sprague-Dawley rats were anesthetized with a mixture of 70 % N_2O and 30 % O_2 The left femoral artery was cannulated for recording of mean arterial blood pressure (MABP) and for measurements of P CO_2 and P O_2 The adjacent femoral vei was also cannulated and used for injections A rise in MABP by 80-120 mm Hg to disrupt the blood-brain barrier was induced by an intravenous bolus injection of angiotensin amide (Hypertensin CIBA; 50 125 µg/kg in 0 10 0 25 ml) The substance does not in itself cause any extravasation of Evans blue in brain vessels (5) lthough an enhanced permeability through the endothelium has been demonstrated in peripheral vessels (6) An acute ise in MABP of thi magnitude (80-120 mm Hg) i possible to obtain only if the blood pressure is lowered beforehand (see Table I) In this study by dihydralazine methanesulphate (Nepresol CIBA; 800 µg/kg in 0 5 ml i 5-10 min before angiotensin) which lowers the tone of vascular smooth musculature The compound has not been shown to change vascular permeability nor does it interfere with the perivascular sympathetic innervation or with the autoregulation of the cerebral blood flow

A patchy and dot-like extravasation of Evans blue (1 ml of 2 % solution given i about 10 min beforehand) was noted to similar extent in surface structures of both hemispheres (Table I) The barrier damage is believed to be caused by pressure-forced overdistension of the vessel walls (7) One to three days after unilateral sympathectomy on acute rise in systemic blood pressure resulted in more marked barrier damage on the sympathectomized ide than on the contralateral side of the brain On the other hand marked acute

Table I. Effect of acute systemic hypertension on blood-brain barrier function as shown by the degree of [illegible] blue-albumin extravasation, which [illegible] expressed subjectively from 0 (no extravasation) to +++ (marked patchy and dot-like extravasation). The hypertension was induced by angiotensin [illegible] preceded by dihydralazine [illegible]. Values on mean arterial blood pressure (MABP) are means ± S.E.M., [illegible] number of animals

Treatment	n	Initial MABP (mm Hg)	MABP after dihydralazine (mm Hg)	MABP increase after angiotensin (mm Hg)	Blood-brain barrier lesions, right side +++	++		Blood-brain barrier lesions, left side +++	++		
Moderate acute hypertension											
control	20	147 2	88 2	91		0				4	
right sympathectomy	12	144	89	94 3		1				3	3
Marked acute hypertension											
control	11	143	88	2				0	3		
right sympathetic stimulation		143	89 1	1			3		1	1	7

rise in systemic blood pressure during electrical stimulation (30 V 15 Hz, 1 msec pulse duration) of the right cervical sympathetic chain below the superior cervical ganglion was associated with only minor if any barrier damage on the stimulated side (Table I)

The results support the view that the cerebrovascular sympathetic nerves protect the cerebral circulation during acute hypertension not only with regard to the limits of autoregulation but also in terms of barrier disruption It is probable that afte cervical sympathectomy the wall of the resistance vessels becomes less well prevented from overdistension when the systemic blood pressure is acutely rised

The passage of noradrenaline and inulin from the cerebrovascular circulation into the brain (vessel wall and parenchyma) was studied quantitatively and regionally by modification of Oldendorf technique (8) for determination of brain uptake index (BUI) The index was determined in sympathectomized rats (3 days fter excision of the superior cervical ganglion) and compared with non- or sham-operated controls For noradrenaline BUI was enhanced about two fold (Table II) The uptake index for an inert substance inulin was similarly enhanced after sympathectomy (Table II) indicating that the increased uptake was not merely due to change in local demand of neurotransmitter but more likely reflected a true impairment of blood brain barrier function Further in 5 out of 10 othe animals

Evans blue a discrete patchy xtravasation of the Evans blue lbumin comple was noticed in cortical structures on the sympathectomized side Since the anesthesia obtained by the N_2O/O_2 mi ture is light it is possible that the cannulated and tracheotomized animal is in state of tress which is supported by the fairly high resting level of MABP in these animals (see Table I) Therefore one possible xplanation for the findings at the level of the blood brain barrier is that the elevation in blood pressure occurring during

Table II. Brain uptake index (BUI) for noradrenaline and inulin [illegible] standard [illegible] of [illegible] rats. Values are mean [illegible] number of animals. Student [illegible] t-test comparison between unoperated and operated [illegible] [illegible] [illegible] [illegible] [illegible] non-significant

	Experimental	Frontal cortex	Parietal cortex	Occipital cortex	Caudate nucleus	Thalamus
NORADRENALINE	Unoperated animals	48 27	26 44	4,44 45	[illegible]	[illegible] 48
	Sham-operated, [illegible] days	46 18	4,34 31	4,12 [illegible]	3 37 19	[illegible]
	Sympathectomized, 3 days	[illegible] .32**	[illegible] .[illegible]	[illegible]	52 [illegible]***	[illegible]***
	Sympathectomized, [illegible] month	[illegible]	[illegible]	[illegible]	4,[illegible] 39	4,37 47
INULIN	Unoperated animals	1 [illegible]	.37 [illegible]	78 [illegible]	47 [illegible]	78 19
	Sympathectomized, 3 days	[illegible] 14***	[illegible]**	73 [illegible]**	[illegible]**	2,78 **

manipulation with the animal is sufficiently prominent to cause overdistension of the walls of the resistance vessels when they are devoid of their sympathetic innervation. Another possibility that may have to be taken into consideration is that the barrier functions of the capillary endothelium which appears to have contractil properties (9) are directly influenced by the nerve endings demonstrated by lectron microscopy i lose approximation to the capillary wall (10).

Conclusions The cerebrovascular sympathetic nerves are important for the maintenance of normal blood brain barrier functions in ituations with sudden marked levations of the blood pressure causing an overdistension of the vessel wall

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IMMUNOHISTOCHEMICAL DEMONSTRATION OF ACTIN AND MYOSIN IN BRAIN CAPILLARIES

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There is evidence especially from lectron microscopi studies (1) that brain capillaries are innervated by non-myelinated probably adrenergic, nerve terminals In order to show whether such nerves have vasomotor function at the capillary level primary requirement would be the demonstration of contractile proteins in the capillary endothelium We have for the first time accomplished this by the use of specifi antisera to highly purified fractions of smooth muscle actin and myosin applied to immunofluorescence histochemistry

Sections from rat hypothalamus and cerebellum were prepared in cryostat They were incubated for 30 min at room temperature with specifi gamma-globulin-enriched rabbit antibodies and corresponding controls as follows ( ) antiserum raised against purified myosi from chicken gizzard smooth muscl (b) antiserum to acti purified from aceton powder of chicken gizzard muscle ( ) antibody to the smooth muscl myosi previously adsorbed to chicken gizzard myosin (d) the same antibody adsorbed to striated muscle (pectoralis) myosin and ( ) normal non-immune rabbit gamma-globulin After washing, fluorescein-labelled sheep anti-rabbit immuno-globulin was added Further controls were incubated with this second antibody lone Biochemical and technical details are found lsewhere (2,3) Adjacent sections (from animal receiving L-dopa) were treated according to the glyoxylic acid method for the demonstration of dopamine This is formed in high concentrations from the L-dopa which is selectively trapped i the capillary walls by an enzymati blood-brain barrier mechanism (4)

Incubation of brain sections with spe ific antibodies against smooth muscle myosin followed by fluorescein-labelled anti-gamma-globuli sera gave intense fluorescence in the smooth muscle wall of pial arteries and arterioles The fluorescent vessels issued branches into the hypothalamic and cerebellar parenchyma The parenchyma contained a network of myosin-fluorescent capillaries (Fig 1) whose identity could be established by comparing with adjacent sections showing capillary dopamine fluorescence after administration of L-dopa (4) The fluorescence intensity in the capillary walls was lower than that of the precapillary vessels. The immunofluorescence was located both in pericytes an ndo-



Fig 1 Fluorescence photomicrographs showing myosin immunoreactivity in the wall of capillaries (some indicated by arrows) and arterioles (A) of rat s hypothalamus Magnification 125 X.

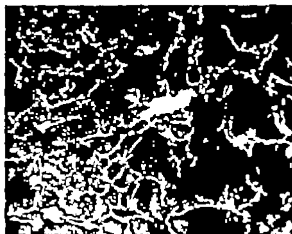


Fig 2 Arteriolar vessel (A) and sinusoid capillaries displaying myosin-specific fluorescence in the fasciculate zone of the rat adrenal cort Arrows point to some of the fluorescent endothelial cells Magnification 250 X

thelial cells Also the venules and veins exhibited a myosin fluorescence in their walls of the same intensity as that seen in the capillary wall Sections incubated with anti-actin showed imilar fluorescence distribution in the vessel including the capillary wall Incubation with myosin antibodies previously adsorbed to striated muscle myosin gave more distinct fluorescence than that obtained with myosin antibodies lone and the greenish background was reduced Previous adsorption t smooth muscle myosin compl tely extinguished the fluorescence Control sections incubated with ither normal non-immune gamma-globulin or the second fluorescein-labelled antibody were completely negative

The presence of actin and myosin has been confirmed in vessels of the adrenal gland (Fig 2) indicating that lso peripheral capillaries have contractil properties The findings offer new aspects on the dynamics of low and permeability in certain vascular beds

Conclusion It has for the first time been demonstrated that contractile proteins — actin and myosin — are located in pe icytes and endothelial cells of brain capillaries and also in peripheral capillaries in g the adrenal gland

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CHANGES IN SENSITIVITY OF CEREBRAL VESSELS TO NORADRENALINE AND 5 HYDROXYTRYPTAMINE IN THE PRESENCE OF SUBARACHNOID BLOOD

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Cerebral vasospasm due to the presence of blood i the subarachnoid space following the rupture of an intracranial aneurysm is still baffling probl m confronting the neurosurgeon and the pathophysiology is far from understood (1)

In the present study the change induced by 1 ml autologous blood injected into the subarachnoid space la the orbit was studied in the rabbit basilar artery The blood injection produces angiographically ishbl spasm of the artery During in itro conditions (2) the contractile activity of noradrenaline (NA) and 5 hydroxytryptamine (5-HT) was determined in normal arteries and in arteries xposed to subarachnoidal blood for 3 days Three days after the intracisternal blood injection the NA fluorescence demonstrated by the Falck-Hillarp technique was reduced in the nerves of ll pial arteries at the base of the

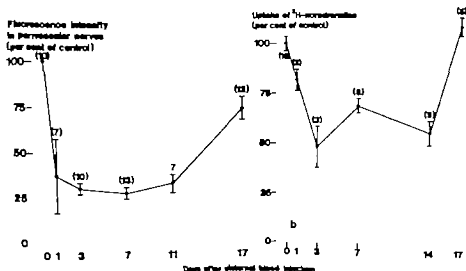


Fig 1 ( ) Fluorescence microscopi estimation of relative fluorescence intensity of the adrenergi nerves (Falck-Hillarp method) in the internal carotid and middle and anterior cerebral arteries at various time intervals following isternal injection of blood Number of animals examined within parenthesis (b) Relative uptake of ³H-noradrenaline in middl and anterior cerebral arteries incubated during 10 min at 37 °C in an amine concentration of 10^{-7} M. Number of animal from which tissues were obtained within parenthesis. Day 0 represents samples from untreated animals

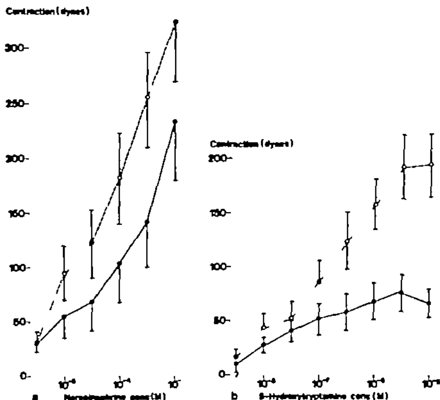


Fig 2 Dose-response curves showing the relative in vitro sensitivity to noradrenaline (a) and 5-hydroxytryptamine (b) in normal rabbit basilar arteries (filled circles) and in arteries from animals treated with intracisternal blood 3 days before the test (open circles) The curves represent mean values ± S E M. of 10 animals

brain (Fig 1) In accordance with this the chemically determined vascular concentration of NA was reduced Concomitantly the capacity of the perivascular nerves to take up and retain ^{3}H NA was markedly impaired (Fig 1b) A normalization was seen after about 2 weeks

Cumulative dose-response curves were obtained for NA and 5-HT (3); in non-treated vessel the ED_{50} was lower for 5 HT whereas the E_{Am} (maximum contraction) was much more pronounced for NA Afte exposure to blood for 3 days the NA dose-response relationship was shifted towards lower concentrations (Fig 2) an ffect lso observed after sympathectomy (4) thus resembling the situation occurring during denervation receptor supersensitivity The ituation with the dose-response curve of 5-HT was different in that the maximum contractile effect (E_{Am}) was increased by about 4 5 times (Fig 2b) This potentiation of the 5-HT contraction by subarachnoidal blood is till unclear It can be assumed that these

increases in amine sensiti ity play role in the development of cerebral vasospasm following subarachnoidal bleeding

Conclusion Subarachnoidal blood — mimicking subarachnoidal hemorrhage — denervates pial vessels and increases thei sensitivity to both noradrenaline and serotonin

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HISTOCHEMICAL, ULTRASTRUCTURAL AND FUNCTIONAL EVIDENCE FOR A NEUROGENIC CONTROL OF CEREBROSPINAL FLUID PRODUCTION FROM THE CHOROID PLEXUS

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The mammalian choroid plexus is highly vascularized villous structure covered with single layer of cuboidal epithelial cells It is present in all four ventricles of the brain and it constitutes the major site for the bulk production of cerebrospinal fluid (CSF) This is formed by an active secretory process at rate that varies considerably from species to species but which is rather constant when expressed as fraction of total CSF volume or on the basis of plexus weight approximately 0 5 % of total CSF volume is replaced by newly formed fl id every minute (4 5) A century ago the histological studies of Benedikt (1) suggested the presence of nerves in the plexuses but in pite of these and later confirmatory morphological evidence (3 17) the possibility of nervous influence on the CSF production has not hitherto been seriously investigated

INNERVATION OF THE CHOROID PLEXUS

Histochemistry of Adrenergic Nerves

Using the Falck Hillarp histofluorescence technique (2) we have shown that the mammalian choroid plexuses receive an ample supply of adrenergic nerves in all ventricles particularly that of the third (Fig 1) though there is rather great variation in the innervation density between different animal species (8) The adrenergic fibres form networks around small blood vessel belonging both to the arterial and venous systems and the fluorescent terminals run in between the epithelium and the underlying vascular wall suggesting that both structures are supplied The nerves originate from the superior cervical sympatheti ganglia (with the exception of few fibres in the plexus from the fourth ventricle as seen in rabbits obviously arising from lower ganglia) This has been demonstrated both histochemically (6 8) and by fluorometric measurement of noradrenaline (10) in denervation experiments

Ontogenetically (Table I) the first adrenergic nerves (of the rabbit) appear in the plexus of the third ventricle around birth Somewhat later around the 5th postnatal day

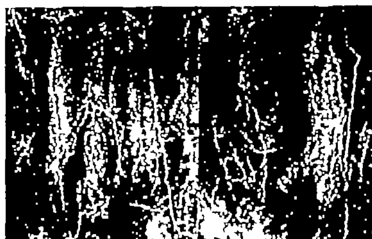


Fig 1 Fluorescence photomicrograph of a whole mount of the choroid plexus from the third ventricle of a rabbit formaldehyde reaction Numerous delicate adrenergi axon terminals form a network in the parenchyma among the epithelial cells Autofluorescent cells and granules are lso seen X 55

such nerves are lso see in the lateral plexuses while that of the fourth ventri le does not receive histochemically isible adrenergic nerves until about 2 weeks post partum. The nerve supply of the whole system of plexuses reaches its full development at approximat ly the age of 3 weeks

Histochemistry of Cholinergic Nerves

The mammalian choroid plexus is also supplied with cholinergi nerves (15) which have bee visualized by the histochemical cholinesterase technique following inhibition of pseudocholinesterase (12, 13) The pl xuses of all ventricles receive acetylcholinesterase-contai ing nerves which ar associated not only with the choroidal blood vessels but also

Table

Development of adrenergic innervation [illegible] Hillarp fluorescence histochemistry in rabbit choroid plexus

Stage / Region ventricle	Prenatally	Birth	[illegible] days	10 days	2 weeks p.p.	3 weeks
Third		very few	moderate	numerous	numerous	fully developed
Lateral	0		very few	numerous	numerous	fully developed
Fourth	0			0	very few	fully developed

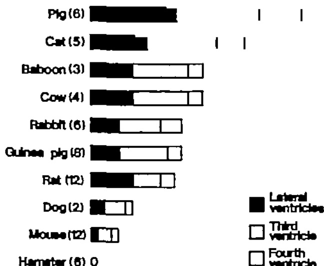


Fig 2 Comparison of the cholinergi nerve supply estimated by microscopy of choroid plexuses from various species Arbitrary units from 1 (scattered nerves) to 6 (extensive supply) The ventri les from which the plexuses were removed are identified to the ight with the 1-unit symbol The hamster plexuses were devoid of histochemically visible nerves Numbers of animals analyzed are indicated within parentheses.

with the plexus epitheli l cells in the same manner as nerves belonging to the adrenergic system The plexus of the third ventri le i usually best supplied with cholinergi fibres whereas that of the fourth ventri le hardly contains any stained nerves at ll The most welldeveloped cholinergi innervation is found in plexus tissue from pig and cat; the mouse and dog plexuses on the other hand receive comparatively few nerve fibres (Fig 2)

Immunohistochemistry of Peptidergic Nerves

It has recently been shown that Vasoactive Intestinal Polypeptide (VIP) potent hypotensive and dilatory agent is present in system of nerves which innervate brain vessels in much the same manner as the autonomi fibres (14) Further immunofluorescence studies have shown that thi type of peptidergi nerves lso supply the choroid plexus Thus substantial number of delicate fibres with perivascular orientation have been found in plexuses from pig cow cat and, to certain extent lso rabbit Isolated twigs are occasionally seen to run in between the wall of small blood vessels and the overlying epithelium



Fig 3 Electron micrograph of rabbit choroid plexus near the free border Small axon bundle near small arteriole (A) enclosed by singl smooth muscle cell (M) and the basal portions of epithelial cell (E) The structures are separated from each other by basement membranes The axons cont in mitochondria neurotubuli large (appro imately 1000 Å in diamete) electron dense vesicles and small empty vesicles X 10 700 Insert Detail of partly naked axon varicosity contai ing a mitochondrion and numerous 300 600 Å agranular vesicles and separated from the membrane of an epithelial cell by 300 Å and from that of the vascular smooth muscle cell by 900 Å X 22 500

Ultrastructure of the Innervation

Electron microscopy of cats and rabbits has shown numerous non myelinated nerves running in relation to the wall of small arterioles and to the base of plexus epithelial cells (6) Thick bundles of axons enclosed by Schwann cells and a thin collagenous investment are found in preparations tak near the border of attachment of the plexuses where they accompany small arteri les Further out in the plexus tissue towards the free border the axons are collected in bundles of smalle ize and are located contiguous to the wall of small arterioles as well as to the base of epithelial cell (Fig 3) Near the fringes of the plexus tufts the nerve terminals run singly and devoid of their neurilemmal sheath

The adrenergi nature of some of the nerve terminals could be secured by the presence of lectron dense synaptic vesicles after injection of the "false adrenergic transmitter

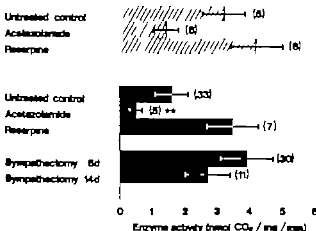


Fig 4 Carbonic anhydrase activity in rabbit choroid plexus from fourth ventricle (dashed bars) and lateral ventricles (black bars) in normal animals and after treatment with acetazolamide (100 mg/kg iv 60 min) reserpine (5 mg/kg ip 4 5 hr) or after sympathectomy (bilateral removal of the superior cervical ganglia) At 14 days after denervation the enzyme activity returns towards normal probably due to denervation supersensitivity of the receptors. Plexus tissue from one animal was used in each determination Mean values $\pm$ SEM, number of determinations within parentheses Comparison of control means with experimental means according to Student's t test: 0 01 < p 0 05, 0 001 p<0 01

5 hydroxydopamine An approximately qual number of terminals contained lectron lucent synaptic vesicles; they were interpreted as the cholinergic terminals visualized at the light microscopi level by the cholinesterase technique Both types of nerves come as close as 200 Å to epithelial cells (Fig 3) where they are located ither contiguous to the base of the cell or cellular processes between adjacent cells or within cellular invagination The distance between the nerve ndings and the smooth muscle cell of the arterioles is in the order of 900 Å (Fig 3) which is characteristic of functioning autonomic innervation of blood vessels

The VIP containing nerves have so far not been identified at the lectron microscopic level

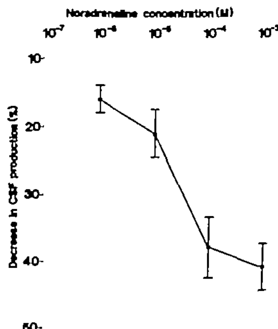


Fig 5 Dose-dependent reduction in bulk CSF production during intraventricular infusion of various concentrations of noradrenaline Mean values ± SEM, 3 4 experiments at each dose level

SYMPATHETIC INFLUENCE ON CHOROID PLEXUS FUNCTION

The structural observations have suggested that the innervation of the choroid plexus may influence functions through an effect both on its vascular bed and on its secretory epithelium Separate studies have therefore been carried out to show the presence of adrenergic receptors in the plexus blood vessels and sympathetic effects on the carbonic anhydrase activity in the plexus epithelium and on the bulk production of CSF

Adrenergic Receptors in Plexus Vessels

The anterior choroidal artery of cows was used as model vessel in the pharmacological analysis of smooth muscle receptors in itro by techniques previously described in detail (7).

During resting conditions of the artery and in the presence of appropriate blocking agents adrenaline noradrenaline phenylephrine and isoprenaline produced dose dependent vascular contraction in the mentioned order of potency However the effect was only about one tenth of that obtained with feline pial vessels of imilar size (9) The reversible

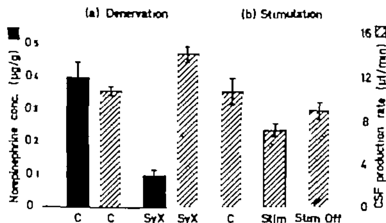


Fig 6 ( ) One week following sympatheti denervation (SyX) of the rabbit choroid plexus there is marked reduction in the norepinephrine concentration concomitant with highly ignificant increase i the rate of CSF production compared with unoperated controls (C) Differences between mean values ($^+$ SEM) according to Student's t-tests p 0 001 i both groups (b) CSF production rate before (C) and during bilateral lectrical stimulation of the superior cervical ganglia (Stim) which markedly reduces the rate of production (the difference based on paired observations was of highest ignificance p< 0 001) After finishing stimulation (Stim Off) there is tendency to normalization of the production rate (Stim vs Stim Off p = 0 01) Bars indicate mean ± SEM.

lpha receptor antagonist phentolamine inhibited the contraction further indicating that it was mediated by alpha adrenergi receptors

The beta adrenergi receptor function was tested on plexus vessel previously given an active tone by prostaglandi F_2 The sympathomimeti relaxation was found to be in the order isoprenaline adrenaline noradrenaline terbutaline The beta receptor nature of the relaxation was confirmed by the competitive inhibition of the isoprenaline induced effect achieved by propranolol

Sympathetic Effects on Carbonic Anhydrase Activity

More direct evidence for true functioning relationship between the sympatheti nerves and the secretory epithelium of the plexus has been obtained in quantitative determinations of carboni anhydrase activity of blood free rabbit plexus by measuring the formation of $^{14}CO_2$ from $Na^{14}HCO_3$ in the presence of plexus homogenate (6) The activity of the enzyme which is essential for the production of CSF is twice as high in the plexus of the fourth ventri le compared to that of the lateral ventricles As xpected treatment of the animals with acetazolamide markedly reduces the enzyme activity in both plexus

regions (Fig 4) Cervical sympathectomy or injection with reserpine significantly increase the acti ity (as measured in the lateral plexuses) by 125 150 % (Fig 4) The findings thus indicate that the sympathetic innervation has an inhibitory action on carbonic anhydrase acti ity and thereby probably on CSF production

Sympatheti Effects on B lk CSF Production

In order to obt in direct measure of the sympatheti nervous influence on the rate of CSF production the ventriculo cisternal perfusion technique of Pappenheimer and collaborators (11 18) utilizing radioactive inulin has been applied to experiments on rabbits (16) According t this technique the rate of bulk CSF production is determined on the basis of radioactive dilution

The animal is anesthetized and artificially ventilated resting prone in a specially made hammock with its head fixed in a metal frame Artificial CSF contai ing inuli ^{14}C carboxyli acid (1 µCi/100 ml) is infused ia cannula into the right lateral ventricle and drained at the same rate through needle placed in the cisterna magna, the outflow being collected at 5 min intervals Infusion pressure is continuously recorded on a Grass P lygraph ia the inflow cannula and Statham pressure transducer Blood gases and systemic blood pressure are followed during the whole xperiment Since diffusion of inulin from the ventricular system is negligibl it follows that any dilution of inulin during the passage through the ventricles results from newly formed inulin free fluid at rate equal to the rat of inflow times the difference between the inulin concentrations in the inflow and outflow divided by the concentration in the outflow (18) The mean production rate of CSF in the untreated rabbit was found to be 10 8 µl/min which is in good agreement with previously published figures (4 5)

Various concentrations of noradrenaline (10^{-8}–10^{-3} M) were administered with the perfusion fl id in order to estimate the effect of the sympathetic neurotransmitter on CSF production (Fig 5) This resulted in dose dependent decrease of CSF production by as much as 42 % at the highest noradrenaline concentration The eff ct of the amine on the rate of CSF formation was compared to the action of acetazolamide which at dose of 100 mg/kg i produced reduction of about 46 % which corresponds to the ffect reported by others (4) Since the choroid plexuses contain high amounts of monoamine idase which can be demonstrated histochemically it may be difficult t define the dose of noradrenaline giving maximum effect due to an unknown degree of breakdown In one series therefore the monoamine o idase inhibitor lolamide was added to the perfusion fl id

at concentration of 10^{-3}M two hours before the noradrenaline administration As could be expected the noradrenaline induced decrease in CSF production became markedly potentiated so that mean reduction of 65 % was now obtained with 10^{-4}M of the amine given in the perfusion fl id

In attempts to relate the action of the xogenous noradrenaline to possibl neurogenl effect on the choroid plexus and CSF production rate the sympathetl trunks in the neck were stimulated lectrically (30 sec on/30 sec off 3 10 V 2 msec pulse duration 15 Hz frequency) Sympatheti stimulation during 1 2 hours (Fig 6) reduced the rate of CSF formation by mean of 32 % compared to the control ituation before stimulation (16) After cessation of stimulation the production rate returned approximately half way (49 %) towards normal during the 1 hour control period f llowing stimulation

In accordance with these observations excision of the superior cervical ganglia had an opposite effect on the CSF production (Fig 6) within 2 days during which lmost all fluorescent nerves in the choroid plexuses of the rabbit are abolished there was a 33 % increase in the rate of bulk CSF production (16)

SUMMARY

Histochemical studies have shown that the rabbit choroid plexus receives well developed adrenergic and cholinergic nerve supply The adrenergi innervation which originates almost entirely from the superior cervical sympathetic ganglia, begins to develop around birth and is fully established 3 weeks later Electron microscopy has shown that the nerve terminals innervate both the plexus arterioles and its secretory epithelium The sympathetic fibres have been shown to influence the plexus epithelium as reflected by thei effect on its carbonic anhydrase activity They appear to have an inhibitory effect on the bulk CSF production as revealed by lectrical nerve stimulation and denervation experiments The control seems to be excerted primarily on the plexus epithelium though some of the effect may lso be associated with alterations i choroid plexus blood flow

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Section III

PERIPHERAL AUTONOMIC INNERVATION

WHAT FUNCTION HAVE CHOLINERGIC NERVES IN THE SMOOTH MUSCLE OF THE MALE GENITAL TRACT?

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The essentially adrenergic nature of the motor innervation of the smooth muscles of the male genital tract is by now fairly well documented (cf ref 1) However there are also strongly cholinesterase positive nerves in the smooth muscle One apparent possible role of cholinergic nerves in the male tract is secretomotor innervation because these nerves are accumulated beneath the secretory cells (2 3) Hence cholinergic nerves may just pass the musculature on their way to the secretory linings However it has been postulated that also part of the motor smooth muscle innervation is cholinergic (eg Robinson (4) It is however also possible that cholinergic and adrenergic fibres interfere with each other because there are intimate connections between these fibres (eg Thoenen et al (5) Here results indicating that this possibility could be one function of cholinergic nerves in the smooth muscle of the male tract will be presented

Material and methods

Isolated strips of the retractor penis muscle and vas deferens of dog were chosen, because in this species the relative proportion of cholinergic nerves (in the muscles in question) seems to be greater than in any other species studied (6 7) The strips were placed in aerated Tyrode solution (35 C) in an organ bath with field stimulating electrode Transmural nerve stimulation (5-10 s every 2 5 min) was performed with pulses of supramaximal strength and frequencies below 15 Hz Contractions were recorded isometrically

Results

In both organs the contractile response to field stimulation was blocked by appropriate concentrations of phenoxybenzamine (1-9 x 10^{-7}M) (Fig 1) phentolamine (3-30 x 10^{-7}M) or guanethidine (1-4 x 10^{-5}M) indicating an adrenergic origin of the motor innervation Acetylcholine (1 x 10^{-7} - 10^{-4}M) contracted the muscles slightly but suppressed the excitatory adrenergic response Physostigmine (1 x 10^{-8} - 10^{-6}M) suppressed this re-

sponse (Fig 2) whil it was restored by scopolamine ($1 \times 10^{-8} - 10^{-6}$M)

Conclusions

Since physostigmine i likely to increase tissue concentrations f acetyl choline released from cholinergi nerves our result support the concept that the function of cholinergi nerves in these smooth muscl i suppression f the adrenergi excitatory neurotransmis ion rather than a direct motor innervation and action T leologically thi may make sense because during sexual arousal secretion should proceed contraction and as mentioned introductorily secretory cells are most likely target cell of cholinergi nerves Thus it could be f some advantage if a secretomotor burst f pulses initially inhibited myomotor transmission

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ELECTRON MICROSCOPY OF ADRENERGIC CHOLINERGIC AND P TYPE NERVES IN THE MYOMETRIUM, AND A SPECIAL KIND OF SYNAPTIC CONTACTS WITH THE SMOOTH MUSCLE CELLS

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The adrenergic innervation of the myometrium has been well elucidated in numerous species — including man — primarily by fluorescence histochemistry and the origi and distribution of the nerves have been larified on the basis of denervation experiments combined with chemical determinations of norepinephrine (1) In the cat about two thirds of the post-ganglionic adrenergic fibres in the myometrium run in the hypogastric nerves whereas the remainder originates in peripheral ganglion formations located in the utero vaginal junction (2) thus belonging to the system of short adrenergic neurons (1)

However surprisingly little has been done to larify at the lectronmicroscopi level the structural basis for functional relation between the adrenergi nerve terminals and uterine non vascular smooth muscle cells

Material and methods

Five adult anestrus cats were used (the cyclic phase was determined by microscopic examination of stained vaginal smears) After pretreatment with 5 hydroxydopamine (5 OHDA; 200 mg/kg i p twice daily for 3 days) the animals were anesthetized by an i p injection of nembutal (30 mg/kg body weight) the chest was quickly opened and cannula inserted through the left ventricle into the ascending aort where the tip was fixed by ligature Fixation was carried out by perfusion with 2.5 % glutaraldehyde solution in 0 1 M phosphate buffer (pH 7 2) for 15 minutes Small pieces from the uterine horns as well as from the cervix isthmus region were dissected out and immersed in the same fixative for another hour The specimens were postfixed in 2 % osmium tetroxide in the same buffer and embedded in araldite (Durcopan ACM; Fluka AG) or Vestopal W They were contrasted en bloc with uranyl acetate before embedding Semithin sections were cut on Pyramitome (LKB) and stained with methylene blue for orientation Ultrathin sections of silver gold interference colour were cut on LKB Ultrotome and placed on formvar coated copper grids If necessary the sections were stained by lead citrat The preparations were



Fig 1 Pedicular process filled with myofilaments extending from smooth muscle cell and seen in close contact (approximat ly 20 nm) with two axon terminals partly denuded of their Schwann cell enclosure X 25 000

xamined in Siemens Elmiskop 1 A or a Philips EM300 electron microscope

Results

On the basis of the characteristics of the synapti vesicles three types of axon varicosities were easily recognized (see further Discussion): ( ) The majority of the axons contained numerous small vesicles (approximately 50 nm in diameter) with highly osmiophili dense core together with large vesi les (100 150 nm in diameter) whose content was much less osmiophilic Such axons are classified as adrenergic (b) Some axons contained only empty small together with a few large vesicles of the same kind as above These axons are probably cholinergi ( ) Besides the adrenergi and cholinergic nerves a third type of axon could be distinguished It was identified by the presence of numerous vesicles having diameters ranging betwee 100- 200 nm and containing a moderately electron dense core sometimes of granular structure filling the entire vesicle In addition the varicosities contained smaller vesi les 50 70 nm in diameter sometimes with a highly lectron dense core These axons too came in lose contact (approximately 20 nm) with the membrane of smooth muscle cells though only in the form of simple appositions The varicosities may belong to a different system usually designated as p terminals

In the immediate icinity of a smooth muscle bundle the varicosities were seen to be partly or wholly denuded of their Schwann cell envelope The distance between the muscle cell membrane and that of the nerve varicosity was about 100 nm the space being occupied

only by the basement membranes of the axon and the muscle cell

The adrenergi axon terminals were seen to establish two kinds of lose contacts lth the effector cells ( ) Most often the naked varicosity simply approached the membrane of the smooth muscle cell to distance of 20 25 nm No membrane spec lizations were observed in these areas (b) In certain areas of the inner and outer muscle layers more specialized connection between muscle cells and nerve terminals was found (Fig 1) The mus le cells extended small stout processes converging onto small nerve fibre Such pr jection sometimes pedicular l shape contained large number of myofilaments (Fig 1) The distance between adjacent membranes was in the order of 20– 25 nm and no thickening of the membranes was seen

Discussion

The use of 5– OHDA as a marker of adrenergi nerves on the ltrastructural level is well established (3) On the basis of the 5 OHDA treatment it was found that the f line uterus contains large number of adrenergi nerves In accordance with previous fluorescence histochemical observations (2) and smaller number of non adrenergic fibres probably cholinergic (parasympathetic) The third type of axon that could be distinguished did not conform, in the appearance of the vesicles with either adrenergi or cholinergic axons These axons resembled those designated as p terminal (4) or purinergic nerves (5)

In the myometrial smooth musculature of the cat two types of earlier described synapses were shown to xist: one type where the adrenergic varicosity comes in lose apposition (20– 25 nm) to the smooth muscle cell with no specialization ither on the neural or muscular side This kind of neuromuscular contact has been described in many tissues from various animal species (6) The other type where the distance between the axon varicosity and the smooth muscle cell is about 100 nm, occurs mainly in neuro muscular contacts of blood vessels and in the gut (6) Also third and not previously described neuro–muscular contact was found Here the muscle fibres were seen to advance stout irregular processes onto adrenergi varicosities the distance between adjacent membranes being in the order of 20– 25 nm These muscular processes seemed to be specialized in the sense that they contained condensation of myofilaments It is possible that the specialized neuro muscular contacts may constitute the directly innervated or "key cells which according to Burnstock and Iwayama (7) is characteristic feature in well innervated organs such as the guinea pig vas deferens cat urinary bladder the pupillary constrictor and the nictitating membrane

Summary

In the cat uterus three types of axons have bee identified by electron microscopy on the basis of the appearance of the synaptic vesicles in the axon varicosities following treatment with 5 hydroxydopamine: (1) Most axons were adrenergic with numerous small osmiophilic vesicles of appro imately 50 nm diamete together with large (100-150 nm in diameter) granules (2) Some axons contained only empty small together with the large granules; these are probably cholinergic (3) A small number of the axon varicosities had only large sized vesicles with a moderat ly lectron-dense content They are interpreted as a polypeptide type of nerves (p terminals") Close appositions at 25 nm distance were sometimes found between the varicosities A specialized neuro muscular connection was found: the muscle cells at "the end of muscle bundles xtended small stout processes containing numerous myofilaments onto a small nerve bundle the distance being 20-25 nm

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REDUCED TYROSINE HYDROXYLASE ACTIVITY IN GUINEA-PIG UTERUS DURING PREGNANCY

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With combination of fluorescence histochemical and fluorometri techniques it has been demonstrated that the myometrium of the guinea-pig uterus receives rich supply of adrenergic nerve fibres, mainly arising from the paracervical ganglia (short adrenergi neurons) and the hypogastric nerves (1). During the end of pregnancy the neuronal fluorescence as well as the uterine noradrenaline disappears (2). I animals with unilateral pregnancy the reduction occurs also in the uterine horn that is not enlarged and distended by the presence of fetuses (2). It has therefore been suggested that these marked changes in the transmitter content which occur xclusively in the reproductive tract may be caused by humoral factors (3). After delivery the noradrenaline content measured in the whole uterus, slowly returns to non-pregnant level which are attained within 6 months (4).

The biochemical mechanisms underlying these marked lterations in the function of the uterine adrenergi nerves are still unknown. Several aspects of the transmitter metabolism may have been implicated such as changes in its rate of synthesis release and/or breakdown within the uterine adrenergi nerves. Recent observations have shown that iso structural degeneration of the uterine adrenergi nerves particularly in the distended portions of the uterine wall is involved in the reduction of the noradrenaline level during pregnancy (5).

The aim of the present study has been to investigate the synthesis of neuronal noradrenaline in the guinea-pig uterus during and after pregnancy. For this purpose the activity of the enzyme tyrosine hydroxylase was measured in order to search for possible relationship between the altered transmitter level and changes in the activity of the enzyme which is rate-limiting step in the noradrenaline synthesis.

Material and Methods

The material consisted of total of 59 female guinea-pigs of mixed strain. After caging with males, animal from three different periods of pregnancy were selected by palpation

mating date and after killing under light anesthesia the day of pregnancy was checked by measurement of mean weight and crown-rump length of the fetuses (6,7) The three groups were at 20-25 30-40 and 60 65 days of pregnancy They were further divided into two sub-groups: animals with bilateral and animals with unilateral pregnancy Animals were lso studied 3 weeks 12 weeks or 6 months after delivery Abdominal xploration immediately post partum had shown that the horn(s) from which tissues were obtained had contained fetuses during pregnancy Five non-pregnant animal served as controls

Immediately after sacrificing the animal the uterine horns were dissected out weighed, frozen and stored in deep freezer until further processing The entire horn was homogenized in the presence of Triton X 100 and an liquot was taken for assay The tyrosine hydroxylase (TH) activity was measured according to the method of Hendry and Iversen (8) determining the formation of ^{3}H-3 4-dihydroxyphenylalanine (DOPA) from ^{3}H tyrosine In tissue homogenates In the determinations the TH activity is obtained as pM (picomoles) of DOPA formed per mg uterine tissue during one hour Where there are differences i uterine weight between various endocrine states the TH activity is lso xpressed as pM of DOPA formed per uterine horn during one hour

The Student s t-test was used in the statistical evaluation f differences in the mean values of uterine TH activity

Results

The results from the determinations of uterine TH activity in the different experimental groups are summarized in Table 1 The TH activity in the various pregnancy groups and post partum groups are compared to the TH activity in non-pregnant animals

F tus-containing uterine horn during pregnancy

At 20-25 days of pregnancy there was an appro imately 50 % reduction of the total TH act-i ity ($p < 0.01$) At 30-40 days of pregnancy the activity was further decreased ($p < 0.001$) and at 60-65 days of pregnancy almost no measurabl TH acti ity was found Since the decrease i total TH activity during pregnancy was associated with an increment in organ weight the reduction i TH activity per mg tissue was even more pronounced

^{3}H 2 3 side chain L-tyrosine

Table 1

Tyrosine hydroxylase activity in uterine horns from non-pregnant pregnant and puerperal guinea-pigs The activity of the enzyme is expressed as picomoles (pM) ^{3}H 3 4-dihydroxy-phenyl lanine (^{3}H-DOPA) formed per hour 1 each mg tissue or 1 the entire uterine horn

Stage	Number of determinations	Organ wet weight (mg)	Tyrosine hydroxylase activity (^{3}H-DOPA formed)	
			pM mg^{1} h^{1}	pM per uterine horn per h
Non-pregnant uterine horn	5	300 3^{+}70 5	1 132^{+}0,147	307 9^{+}43 9
F tus-containing horn				
20 25 days pregnancy	4	752 6^{+}89 0	0 204^{+}0 048	141 6^{+}13 9
30-40 days pregnancy	11	2100,0^{+}315 5	0 048^{+}0 012	83 2$\pm$22 8
60-65 days pregnancy	5	7041 2^{+}605 4	non-measurable	non-measurable
Non-fetus containing horn				
20-25 days pregnancy	4	231 9^{+}26 7	0 650^{+}0 027	148 9$\pm$11 9
30-40 days pregnancy	5	393 8^{+}67 5	0,251^{+}0 066	81 1^{+}24 6
60-65 days pregnancy	3	954 4^{+}151 5	0 045^{+}0 022	37 2^{+}16 2
Uterine horn at 3 weeks post partum	8	495 6^{+}53 2	0 066^{+}0 010	32 5^{+}5 2
12 weeks post partum	5	773 6^{+}52,6	0 040^{+}0 008	30 5^{+}5 6
6 months post partum	8	743 6^{+}56 2	0 163^{+}0 027	124 1^{+}23 1

Non-fetus containing (empty) uterine horn during pregnancy

I comparison with uterine horn from non-pregnant animals also the empty uterine horn of pregnant guinea-pigs showed a reduction in total TH acti ity At 20 25 days of pregnancy the reduction amounted about 50 % ($p < 0.02$) At 30–40 days of pregnancy the decrease was somewhat more than 70 % ($p < 0.005$) and at 60–65 days of pregnancy the reduction was about 90 % ($p < 0.005$) The TH activity as expressed per mg tissue was lso reduced

Post partum

At 3 and 12 weeks after delivery the total TH activity (in the uterine horns that had contained fetuses) had increased as compared to the non-measurable values at 60–65 days of pregnancy It was however still only 10 % of the level in non-pregnant animals ($p < 0.001$) At 6 months fte delivery it had further increased and now reached about 40 % of the enzyme acti ity in the non-pregnant animals ($p < 0.005$)

Discussion

The guinea-pig ut rus receives a well developed supply of adrenergic nerves, which innervate the myometrial smooth musculature as well as the uterine vascular bed (1 3 9) These uterine neurons are unique i the sense that their transmitter content varies during, for example pregnancy (2) Thus during the end of pregnancy there is a marked decrease in the number of nerves xhibiting a formaldehyde-induced noradrenaline fluorescence Comparison of the two uterine horns in unilaterally pregnant animals has revealed that this conspicuous change in the adrenergi nerves occurs whether the uterine horn contains f tuses or not (5) Although the uterine horn containing f tuses is distended and much larger than the contralateral horn lacking fetuses the chemically determined noradrenaline content shows similarly pronounced reduction in both uterine horns during late pregnancy; however the course of disappearance is different in the two horns (2) It is therefore unlikely that only mechanical factors should be responsible for the disappearance of adrenergic transmitter in the uterine nerves Recent observations on the capacity of uterine adrenergic nerves to accumulate the noradrenaline analogue -methyl-noradrenaline after disappearance of the endogenous transmitter in combination with silver impregnation studies have indicated that the noradrenaline reduction in the fetus-contai ing horn may mainly be due to nerve degeneration (at least during late pregnancy) whereas i the empty horn noradrenaline is lost principally from intact nerve fibres (4 5)

The present results show that reduced synthesis of noradrenaline is compatible with the reduced level of transmitter during advancing pregnancy Since TH is rate-limiting in the noradrenaline synthesis, the activity of this enzyme was chosen as parameter The TH activity was reduced to very low level at the end of pregnancy and in fetus-containing horns from late pregnancy no detectable ^{3}H-DOPA activity was found (1 the total number of counts was below that of the blank sample without tissue) This holds true both when the enzyme activity is expressed on organ weight basi and as total activity in the uterine horn Expressing the total TH values compensates for the pronounced increase i organ weight during pregnancy Compared to the empty horn the activity per mg tissue was reduced to greater extent in the fetus-containing horn where non-measurable level were found at the end of pregnancy The same tendency holds true lso when the enzyme activity is xpressed in terms of total activity per uterine horn Due to the very marked enlargement of the fetus-containing uterine horn, the actual total enzyme activity in the tissue is difficult to establish This possible difference would be in accordance with the above-mentioned view that structural disintegration of the adrenergi nerves i involved in the fetus-containing horn during its enlargement

After delivery there i slow progressive recovery of noradrenaline in uterine nerves during the post partum period (4) This is in general agreement with the slowly returning TH activity which however is still not more than 40 % of non-pregnant control values as late as 6 months after delivery

Acknowledgement

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Summary

In previous series of investigations it has been shown that uterine noradrenaline in the guinea-pig is markedly reduced during advancing pregnancy reaching near-zero levels at the time of parturition After delivery noradrenaline slowly returns towards non-pregnant levels In animals with unilateral pregnancy similar noradrenaline reduction occurs also in the horn lacking fetuses. This indicates that the alteration in the neuronal transmitte is the consequence not only of mechanical factors but probably also of some humoral mechanism

In the present study the uterine tyrosine hydroxylase activity was determined in non-pregnant guinea-pigs and in animals at various stages of pregnancy as well as after delivery It was found that the enzyme activity was gradually reduced during pregnancy in both the horn containing f tuses and in the horn lacking f tuses At the time of delivery non-measurable levels were found in the f tus-containing horn and the activity was reduced by about 90 % in the non-fetus contai ing horn During the post partum period the enzyme activity increased very slowly to reach about 40 % of non-pregnant values by 6 months after parturition

It is suggested that a reduction of the noradrenaline synthesis partly associated with an actual degeneration of the adrenergic axons, is factor involved in the fall of uterine noradrenaline seen during pregnancy

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ORGANIZATION AND FUNCTION OF THE SYMPATHETIC INNERVATION OF HUMAN LIVER

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The relations between the sympatheti nervous system and the liver cells have been dealt with i both morphological and functional studies (1 2 3 4 5) Physiological experiments on calves dogs and cats have shown the importance of the sympatheti nerves in controlling the hepati glucose release not only during direct nerve timulation (1 2) but also during reflex ituations such as bleeding (4) The existence of sympatheti nerves innervating liver parenchymal cells would thus be expected However Ungvary and Donath (3) reported that in series of laboratory animals which they investigated they could not find any adrenergi innervation in the liver except that confined to blood vessels These negative findings have recently been confirmed by Skaaring and Bersing (6) On the ther hand Yamada (7) observed on the electron microscopical level nerve fibres containing dense core ves les in lose contact with liver cells in mouse liver There thus seems to be discrepancy between the morphological and functional findings concerning the sympathetic innervation of the liver

This study presents the finding of sympatheti innervation of liver parenchymal cells in man Aspects on the functional role of this innervation i glucose regulation is lso given Preliminary reports on some of th findings have been published earlier (8 9 10)

Materials and Methods

Fluorescence microscopy: The liver pieces for the study were obtained from biopsies taken peroperatively All pieces were examined with routine histological stai ings and only liver tissue without pathological changes was included in the study Within 10 min after the biopsies were taken the liver specimens were frozen in propane-propylene mixture at the temperature of liquid itrogen After freeze-drying the preparations were processed for the fluorescence microscopical visualization of biogeni monoamines according to the Falck Hillarp method (11 12)

Chemistry: Norepinephrine (NE) i liver tissue was determined fluorometrically according to the method of Bertler et l (13) and Häggendal (14) Glucose i plasma was analysed with glucose-oxidase method

Nerve stimulation: The hepatic sympathetic nerves were stimulated peroperatively in 7 patients who were laparotomized for gall-bladder disease but were otherwise healthy and had normal liver function and normal fasting blood glucose values The sympathetic nerve fibres around the hepatic artery were dissected free and put on bipolar platinum electrode The nerves were stimulated for 10 min with 20 Hz 50 V and 2 msec Arterial blood samples were withdrawn before during and after the stimulation and analysed for plasma glucose concentration



Fig 1 Fluorescence photomicrograph of normal human liver tissue Numerous green-fluorescent adrenergic nerve terminals are seen in the parenchyma and some fibres are surrounding individual hepatocytes X 180

Results and Comments

Human liver tissue was found to contain varicose nerve terminals which displayed the green formaldehyde-induced fluoresc ence that is characteristic of catecholamines and they were thus classified as adrenergic fibres (Fig 1) The adrenergic parenchymal innervation was rather dense and the terminal varicose fibres were arranged in a pattern giving the impression that most of them ran around lobular units Fibres were also seen to penetrate into the lobuli sometimes surrounding individual hepatocytes (Fig 1) The density of adrenergic fluorescent fibres did not differ markedly between different portions of the liver but they seemed to be unevenly distributed within most microscopic sections examined Apart from this parenchymal innervation we observed a large number of adrenergic nerve fibres running along both portal and hepati blood vessels These results thus clearly indicate

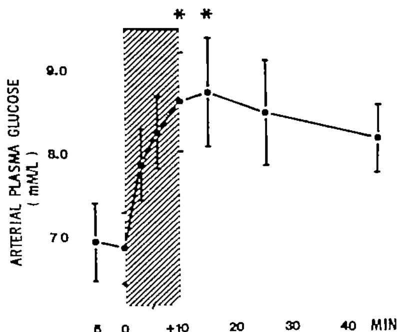


Fig 2 Changes in the arterial plasma glucose concentration in response to stimulation of the hepati sympatheti nerves in man Hatched area represents the stimulation period Mean values + S E.M. of 7 determinations are given Student s t-test difference from control values * = 0 05 > p > 0 01

that human liver tissue receives dense sympatheti innervation which is organized I close contact both to hepatocytes and blood vessel Work in progress confirms these data on the lectron microscopical level (15) The chemical analysis showed high concentrations of norepinephrine in human liver tissue (0 92 + 0 19 µg/g mean value + S E M. of 8 determinations) which gives further support to the view that norepinephrine is the intraneuronal amine in the fluorescent parenchymal nerves

The functional significance of this innervation in the control of blood glucose concentration was studied by stimulating the hepatic sympathetic nerves peroperatively Fig 2 shows that in response to stimulation the arterial plasma glucose concentration increased promptly from control value of about 7 mM/L to peak value of about 9 mM/L 5 ml after cessation of stimulation The difference between the control glucose value and the

stimulation-induced values is statistically significant at 10 and 15 min after the beginning of the stimulation The arterial plasma glucose concentration then gradually returned towards the control level

The present stimulation experiments in man clearly indicate that the hepatic sympathetic nerves induce release of glucose from the hepatocytes This is i accordance with previous findings in animals (1 2) but the magnitude of the increase i plasma glucose is somewhat lower in man than in cat dog pig and calf This difference might at least partly be attributed to the technical problems which are inherent in these human experiments e g the difficulties i dissecting free all sympathetic fibres around the hepatic artery

Acknowledgement This study has been supported by grants from the Magnus Bergvall foundation and from the Swedish Medical Research Council (grant no 04X 56)

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Section IV

AMINE MECHANISMS IN ENDOCRINE SYSTEMS

Acta physiol. scand. Suppl. 452, 1977

THE ADRENERGIC INNERVATION OF THE PITUITARY OF THE EEL *Anguilla anguilla* WITH SPECIAL REFERENCE TO THE CONTROL OF THE PARS INTERMEDIA

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The pituitary of the eel consists of three lobes (Fig 1): the rostral pars distalis (RPD) the proximal pars distalis (PPD) and the neuro-intermediate lobe (NIL) The latter lobe secretes MSH (melanophore-stimulating hormone) the effect of which is to darken the skin by dispersing the pigment of melanophores Neural processes penetrate all parts of the adenohypophysis but the endocrine cells are not directly innervated but separated from the processes by a vascular membrane (1)

Specific formaldehyde-induced fluorescence has been found in the neural processes within all parts of the gland and microspectrofluorimetric analyses have shown that both DA and NA (probably in a fairly equal ratio) are responsible for the fluorescence (2 3) It was also found (2) by using animals which had been adapting for varying times to a white or to a black background that the fluorescence in the NIL varied according to the duration of the adaptation The lowest levels of fluorescence were generally found in animals that were completely adapted to a white background and the highest amounts in those which had been kept on a black background for a long time (about 2 months) or in specimens recently (3-6 hrs) transferred to a white background In the animals which had been adapting to the white background for 3-6 hrs the fluorescent material was evenly distributed in the neural lobe processes while in the animals which were fully adapted to the black background it had coalesced into semicircula structures near the vascular membrane

A possible explanation for the variation i the amount of fluorescent material in relation to background adaptation i that the aminergic nerves re act ve and release transmitter when the animal is on a white background i e when MSH secretion is inhibited Injections of CA-depl ting drugs such as reserpi 6-OHDA and 6-OHDA res lt in a maximal darkening of the skin and i a dis appearance of the fluorescence in the NIL (4 5 2) which gives further support for the i volvement of the CA nerves directly or indirectly in the

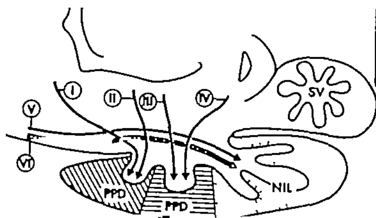


Fig 1 Schematic drawing of the pituitary of the eel indicating its nervous connections RPD rostral pars distalis; PPD proximal pars distalis; NIL neuro-intermediate lobe; SV saccus vasculosus; I V fluorescent tracts; VI neuro-secretory tract Modified from (3)

inhibition of the MSH release The direct action could be by diffusion of transmitter across the vascular membrane that separates the nerve endings from the endocrine cells

The origin of the CA nerves of the pituitary has not been established with certainty However a certain pattern has been established (3) The RPD seems to receive its catecholamine nerves from paired frontal and dorsal tracts (Fig 1 tracts I and II) Also the PPD is innervated from a dorsal direction and some fibres approaching from a posterior direction have also been seen (Fig 1 tracts III and IV both paired) Some of the nerves terminating in the pars distalis probably originate in the various parts of the paraventricular organ (PVO) This comple situated in the walls bordering the third ventricle contains mostly DA and to a lesser extent serotonine The NA component in the pars distalis might originate in the lower brainstem in the autonomous nervous system or in the PVO-accompanying cells i e large NA cells situated laterally to the frontal part of the PVO

The NIL mainly receives its CA nerves via an unpaired median tract (Fig 1 tract V) which is arranged closely around the neurosecretory (NS) tract

(Fig 1 tract VI) More frontally the fluorescent tract seems to become bilateral and more diffuse After a lesion in the base of the pituitary between the PPD and the NIL an accumulation of fluorescent material was seen in this tract accompanied by a disappearance of the fluorescence in the NIL and a darkening of the animal

Lesions in the hypothalamus (Fremberg in preparation) have verified that the secretion of MSH from the pars intermedia is predominantly under inhibitory control of the hypothalamus Furthermore by making selective lesions it was found that the nerves inhibiting the MSH release are situated in a narrow strand close around the NS tract in agreement with the fluorescent tract described above The NS tract is not involved in MSH inhibition

The origin of the inhibitory fluorescent tract is still uncertain From a comparative view the centres inhibiting MSH release might be expected to be situated in the diencephalon or telencephalon So far only three aminergic nuclei have been established here with certainty (3) the PVO the PVO-accompanying cells and the nucleus hypothalami anterior the latter being situated immediately behind the commissura transversa Any of these nuclei may be the source of the CA nerves involved i the inhibition of the MSH release Further work to localize this system more precisely is in progress

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INFLUENCE OF SYMPATHETIC NERVES AMINE RECEPTORS AND ANTI ADRENERGIC DRUGS ON FOLLICULAR CONTRACTILITY AND OVULATION

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The contractile properties of ovarian tissue including the follicular wall have been known for more than half century (1) The nature of the contractil lements involved has, however been matter of controversy ince the histological staining techniques available have not provided suffi ient selectivity for the identification of specifi system of smooth muscle lements in the ovary For this reason it has often been claimed that ovarian and follicular contractility is entirely secondary phenomenon simply related to the contractile activity of smooth musculature in the ovarian vascular bed When electron microscopy was applied to the problem, first by O'Shea (2) it became possible to demonstrate the presence of non-vascular cell structures in the ovary that could be lassified as contractil Also the theca externa of the Graafian follicle contains elements with the ltrastructural characteristics of contractil cells, 1 containing myofilaments, dense bodies, and micropinocytoti vesicles (3 4) Such cells are present in the entire theca externa with the exception of the most attenuated apex region where the follicular rupture occurs Because of the relatively low content of filaments in the smooth muscle-like cells as well as the presence of transition forms between such cell and fibroblasts within the theca xterna it has still been questioned whether they should be regarded as true contractile smooth muscle cell It therefore seemed important to obtain more specific evidence for the contractil nature of the (non-vascular) smooth muscle-like cell in the follicular wall This has recently been achieved by immuno-histochemical demonstration of contractile proteins, actin and myosin in their cytoplasm, as shown i Fig 1 (5,6)

Using fluorescence microscopi techniques for the visualization of the sympathetic neurotransmitter (7 8) it has been possibl to show the presence of well-developed system of non-vascular adrenergi nerve terminals both in the human ovary (3 9) and in ovaries from other mammalian species (for references, see Bahr t al 10) In study focusing on the innervation of the human Graafian follicle it was found that adrenergic nerve terminal are present in the entire wall of the follicle except for the apex region; in direction away from

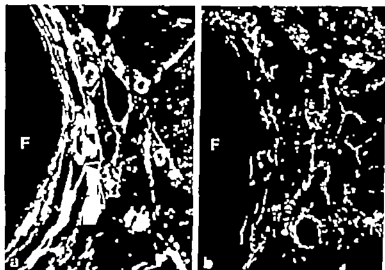


Fig 1 Adjacent sections showing relationship of contractile cell and adrenergi nerves in the theca externa of a large rat follicl (F) and in adjacent stroma including blood vessels ( ) Immunohistochemical demonstration of smooth muscle myosin The theca xterna contains intensely immunofluorescent smooth muscle cell and contractile cells are also found in strands within the stroma and in vessel walls X 105 (b) Adjacent section reacted with glyoxylic acid for isualization of adrenergic nerves. Several adrenergic terminals enter the theca terna to run in between the contractile cells in this layer X 105

this region the number of (non-vascular) nerve terminals increases and is highest in the intraovarian part of the follicl (3)

Attempts have been made to find possibl relationship between the ovarian adrenergic nerve terminals and smooth muscle cell by treating adjacent sections of rat ovaries for immunohistochemistry of contractile proteins and for demonstration of adrenergic nerves by glyoxylic acid histochemistry (6) Comparison of the consecutive sections showed that the sympathetic axon terminals to a large extent ran in among the contractile cell in the follicular theca externa (Fig 1b) At the ltrastructural level the sympathetic nerves could be selectively identified provided the tissue was preincubated in buffer solution containing the false adrenergic transmitter 5-hydroxydopamine which is taken up xclusively by adrenergic nerve terminals rendering the synapti vesicles highly lectron-dense Numerous sympathetic nerve terminals could be seen in the theca xterna of Graafian follicles and the axon varicosities approached the smooth muscle cell with membrane-to-membrane distance of less than 100 nm (3 4 11) indicating that there is functioning neuro muscular relationship in the follicular wall (Fig 2)

In order to obtain more direct evidence that the release of noradrenaline from the local



Fig 2 Electron micrograph from the theca externa of an ovarian follicle from guinea-pig A naked adrenergic nerve terminal (arrow) containing dense-cored characteristic of adrenergic axon vesicles approaches the smooth muscle cells (mc) with neuro-muscular distance of only about 25 m X 8000

adrenergi nerves can induce contraction of the follicular wall transmural lectrical stimulation of isolated strips from human follicl were carried out in vitro (3) When trains of lectrical stimulation (at pulse duration of 0.6-2 0 msec and lasting for 5 sec) were applied, the follicle strip began to contract after short latency to reach maximum soon after cessation of the stimulation followed by rapid relaxation The short pulse duration provides selective stimulation of the autonomi nerves without directly activating the smooth muscle cells This was further confirmed by addition of tetrodotoxin which inhibits neural transmission through blockade of the Na^+/K membrane pump mechanism Tetrodotoxi (at concentration f 3 10^{-6}M) in the bath compl tely abolished the contractile response to lectrical field stimulation whil the direct effect of exogenous noradrenaline on the smooth musculature remained unchanged (Fig 3) In another series of tests it was shown that addition of phentolamine (which inhibits the adrenergic -receptors) or bretylium (which blocks the adrenergic nerves) into the bath markedly inhibited the contraction induced by lectrical stimulation Thus the lectrically induced contraction was mediated mainly by the activation of the adrenergi system of autonomic nerves The xperiments prove that sufficient amounts of neurotransmitter can be released during activation of the follicular sympathetic nerves to produce marked contraction of the follicular wall This contraction induced by noradrenaline is associated with dose-dependent increase in intrafollicular pressure which has been observed by direct measurements in isolated human follicles (3)

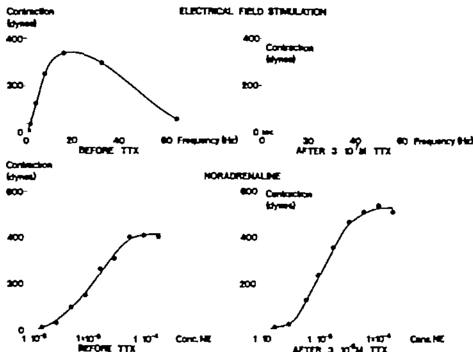


Fig 3 Representative xperiment showing the effect of tetrodotoxin on the response of follicle strip to transmural electric stimulation or noradrenaline Upper curves: Response to electrical stimulation at increasing frequencies before and after application of tetrodotoxin respectively Supramaximal voltage pulse duration 2 ms duration of stimulation periods 5 sec Lower curves: Effect of increasing concentrations of exogenous noradrenaline before and afte application of tetrodotoxin Noradrenaline induces contraction lso in the presence of tetrodoto in confirming that tetrodotoxin has abolished the nerve-induced response selectively whereas the smooth musculature has not been ffected by the drug

The adrenergic receptors mediating the nerve-induced motor activity of the follicular smooth muscles were characterized in pharmacological xperiments utilizing the previously mentioned in itro model (3 12) Full log dose-response curves were plotted for the contractil force developed by the follicular strips fter application of increasing doses of noradrenaline or othe adrenergic agonists The tests were carried out i the presence of ocaine (10^{-6}M) and normetanephrine (10^{-6}M) in the bath to inhibit the neuronal and extraneuronal catecholamine uptak mechanisms respectively allowing for a quantitative evaluation of the responses During the study of contractile receptors lso propranolol (10^{-6}M) was present in the bath to avoid interference with any dilatory β-receptors The order of potency by which the various adrenergic agonists produced follicular contraction was noradrenaline > adrenaline > phenylephrine > isoprenaline indicating that α-receptors mediated the

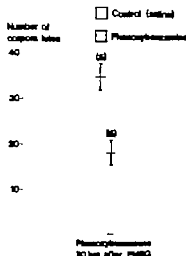


Fig 5 Experiment on inhibition of ovulation in rats. Effect of phenoxybenzoamine (injected locally into the ovarian sac on both sides of immature animals) on the number of corpora lutea induced by previous gonadotropin stimulation. The ovulation rate is markedly reduced in ovaries in which the -adrenergic receptors have been blocked, as compared with matched control animal injected only with saline into the ovarian sac

that some kind of mechanical factor is involved. It is not necessary that such factor is implicated by increasing the follicular pressure to produce rupture of the follicular wall and expulsion of the ovum. It is generally assumed that intrafollicular pressure does not change in association with ovulation. However, it should be noticed that such studies have been performed without knowledge about the presently described adrenergic mechanisms which can indeed increase both follicular wall tension and pressure. Another possibility is that the smooth musculature provides one mechanical basis for the maintenance of the constant intrafollicular pressure claimed to exist during the period preceding follicular rupture. Any of these processes, primarily controlled by the gonadotropins, may be influenced by the sympathetic innervation. The sympathetic nerves might also act on the smooth muscle system in an entirely different way, for example related to those changes in the vascular bed required to produce the ischemia which seems to form part of the rupture mechanism. It may even be that the nerves do not primarily mediate motor function but have trophic influence on the development of the follicular wall or its transformation after ovulation.

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5-HYDROXYTRYPTAMINE-CONTAINING ENTEROCHROMAFFIN CELLS: STORAGE SITE OF SUBSTANCE P

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Substance P was detected by von Euler and Gaddum (1) in extracts of brai and intestine by virtue of its potent hypotensive and gut smooth muscl stimulating effects Recently substance P was isolated and found to be an 11-amino acid peptide (2) By immunohisto-chemistry substance P has been found to have dual distribution i the gut; i system of intrinsic nerves and i endocrine-lik cells scattered i the mucosa (3 4) From the distribution pattern of the immunoreactive endocrine-like cells it was suggested that they belonged to the system of enterochromaffi cells known to be th storage site of 5-hydroxy-tryptamine (5-HT) (cf 5) The recent finding of large amounts of substance P immuno-reactive material in certai 5-HT-producing gut carcinoid tumours (6 7) supports this assumption In order to get direct evidence for the occurrence f substance P immuno-reactivity in enterochromaffin cells tudy was performed on the mouse colon known to be fairly rich in substance P immunoreactive cells (3) Specimens were dissected out and frozen i propane-propylene mixture cooled to the temperature of liquid nitrogen and freeze-dried Some specimens were exposed to diethylpyrocarbonate (DEPC) vapours for 3 hrs at 55°C (8) while thers were treated with formaldehyde vapours for 1 hr at 80°C (standard Falck-Hillarp procedure (9) All specimens were embedded in paraffi in vacuo Sections were cut at 5 μm and placed on albuminized glass slides

Formaldehyde vapour fixation induces strong fluorescence from 5-HT (9) but preserves substance P immunoreactivity only poorly DEPC fixation has previously been found to give an excellent preservation of substance P immunoreactivity (3 4) This fixation does not visualize 5-HT

In order to enabl visualization of both 5-HT and substance P immunoreactivity sequentially in one and the same tissue section two lternatives were tried: 1 Non--deparaffinized sections from DEPC-treated material were exposed to formaldehyde vapours for 2 hrs at 80°C This treatment induced weak to moderate yellow fluorescence typical of 5-HT in the enterochromaffin cells 2 Sections from formaldehyde-treated material were used As could be expected the enterochromaffin cells displayed intense yellow fluorescence due to their content of 5-HT Several sections were examined and photo-graphed They were then processed for the immunohistochemical demonstration of

P using the PAP technique of Sternberger (10) The substance P antiserum (se (kindly supplied by Dr Göran Nilsson Department of Pharmacology Karoli Stockholm Sweden) was used in dilution 1:20 (alternative 1) or 1:10 (alternat controls served sections incubated with antiserum inactivated by the addition c antigen (100 µg of synthetic substance P per ml diluted antiserum)

Both alternatives permitted the subsequent immunohistochemical demonstra substance P However though clearly visible the intensity of immunoreaction treatment with formaldehyde or DEPC plus formaldehyde was weaker than that se sections from material treated with DEPC alone This could at least partly be by using the substance P antiserum in lower dilution As previously shown substan immunoreactivity occurred in nerve fibres predominating i the smooth muscle lay scattered endocrine-like cells in the mucosal epithelium Comparison of photogra 5-HT-fluorescent cells with substance P immunoreactive cells in the same section that the latter cells were identical with 5-HT-containing cells (Fig 1) From n estimate it appears that one out of four enterochromaffin cells in mouse colon disp substance P immunoreacti ity

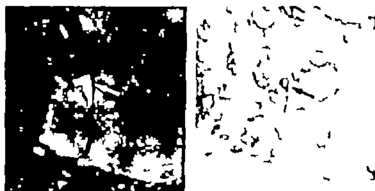


Fig 1

Mouse colon formaldehyde vapour fixation Left: Two adjecent enter cells in crypt epithelium displaying intense yellow fluorescence due to their content of 5-HT Right: Same section immunohistochemical demonstration of substance P (PAP technique) the two enterochromaffin cells (arrow) displays substance P i (X 300)

It can thus be concluded that the substance P cells in the gut mucosa are identical with sub-population of 5-HT-containing enterochromaffin cells. Similar results have recently been obtained by Heitz et al. (11) on rabbit bile duct and human duodenum. However, in contrast to these authors we invariably find substance P immunoreactive cells to contain 5-HT.

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SEROTONIN AS MARKER FOR THE SECRETORY GRANULES IN THE PANCREATIC β-CELL

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The occurrence and function of biogenic amines in endocrine organs have been subject to intensive study since Falck and Hellman (1 2) described formaldehyde induced fluorescence in diff rent cells of the islets of Langerhans. Although some polypeptide hormone-producing cells lack detectable amounts of naturally occurring amines the ability to take up and decarboxylate amine precursors seems to be a general phenomenon (3). Whereas the insulin-producing β-cells from the guinea pig contain easily detectable amounts of amines (2) the detection of natural amines in the mouse β-cells is difficult (4). However these cells readily take up 5-hydroxytryptophan and decarboxylate it to serotonin (5 8). Studies with electron icroscopic histochemistry (9) autoradiography (10) and subcellula fractionation of islet homogenates (11) have suggested that the serotonin is stored within the insulin-contai ing secretory granules of the β cells. The proposed i tragranula localization of serotonin initiated studies on its possible use as a marker for the β-granules and their release of insulin in response to glucose stimulation of the β-cells. Hellman et al (11) used bat ch type of incubations in an attempt to study concomitant release of insulin and serotonin after preloading isl ts with radioactive serotonin. Since there was no parallelism between the amounts of insulin and the radioactivity in the incubation medium in those experiments a more sensit ve approach was tested utilizing the time resolution of a microperifusion system.

β-cell rich pancreatic islets were microdissected from obese-hyperglycemic mice. The islets were preloaded with radioactivity during 120 min incubation a salt-balanced Tris buffe (12) supplemented with 17 μM ^{3}H-5-hydroxytryptophan 1 mM of the monoamine oxidase inhibitor Pargyline and 20 mM glucose. Afte preloading the islets were rinsed for 10 mi in the same medium containing 1 mM Pargyline and 3 mM glucose. The rinsing was followed by perifusion of 10 isl ts in each of two channels of a microperifusion system. The medium used was a Hepes buffered Krebs-Ringer solution (13) supplemented with 1 mM Pargyl ne and 3 or 20 mM glucose as indicated by the black bar in the figures. At the beg nning of perifusion the islets contained about 1 5 mmoles/kg dry weight of 5-hydroxytryptophan equivalents; an amount which decreased by about 60 % during 1 min of perifusion. Analysis of the perifusion medium by means of daisy and microchromatography (6) revealed that more than 90 % of the rad

was released as serotonin justifying the term serotonin efflux In the first experiments islets isolated from fed mice were used since such islets are known to respond readily to glucose stimulation with insulin release The lower curve of Fig 1 shows that glucose stimulated serotonin efflux from this type of islet A parallel experiment (upper curve) was performed in the presence of 1 mM 3-isobutyl 1-methylxanthine (IBMX) which is a potentiator of glucose-stimulated insulin release It is obvious that the glucose effect on serotonin efflux was potentiated by this phosphodiesterase inhibitor

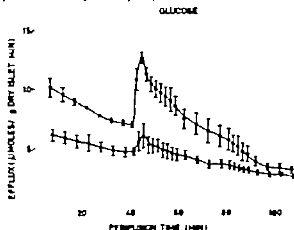


Fig 1

Pancreatic islets isolated from mice starved for a long pe iod of time are characterized by an impairment of glucose-stimulated insulin release (14) Fig 2 shows that glucose has no effect on serotonin efflux when the experi mental animals have been starved for 3 days

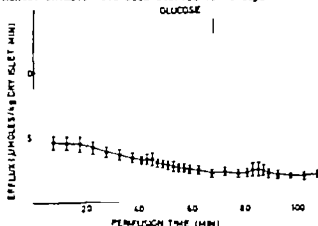
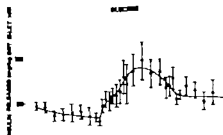


Fig 2



Phosphodiesterase inhibitors are known to restore the glucose effect on insulin release after starvation (14) When 1 mM IBMX was added to the perifusion medium both glucose stimulation of insulin release and serotoni efflux were observed using islets isolated from mice which had been starved for 3 days (Fig 3)

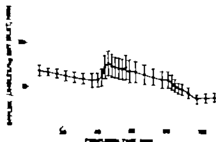


Fig 3

An effective way to abolish glucose-stimulated insulin release is to exclude Ca^{++} from the medium. Fig 4 shows serotonin efflux in the presence of 1 mM IBMX In one of the two parallel experiments (lower curve) no Ca^{++} was added and the medium was supplemented with 0 6 mM EGTA. It is clea that lack of Ca^{++} also inhibited glucose-stimulated serotonin efflux

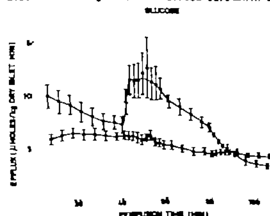


Fig 4

Adrenaline is a potent inhibitor of glucose-stimulated insuli release The lower curve of Fig 5 shows that 2 µg/ l effectively inhibits also the glucose-stimulated serotoni efflux in the presence of 0 1 mM IBMX

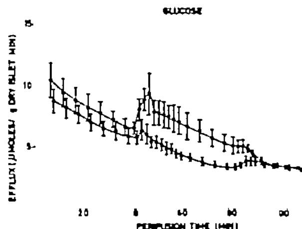


Fig 5

In summary: The present results show a complete parallelism between the effects whether stimulatory or inhibitory on serotonin efflux and insulin release thus supporting the idea of an intragranular localization of serotonin It is suggested that serotonin can be used as a marker for the β-granules and their release of insulin via exocytosis Apart from the suggested insulin associated release of serotonin there is also a serotonin efflux depending on non-exocytotic transport of serotonin across the plasma membrane

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Studies on the Regulation of Metabolism in Human Skeletal Muscle using Intermittent Exercise as an Experimental Model

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STOCKHOLM 1978

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 454

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es on the Regulation of Metabolism in Human Skeletal Muscle using Intermittent Exercise as an Experimental Model

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BIRGITTA ESSÉN

STOCKHOLM 1978

Contents

The thesis is based on the following papers:

I Utilization of blood borne and intramuscular substrates during continuous and intermittent exercise in man B Essén L Hagenfeldt and L Kaijser J Physiol 1977 265 489-506

II Metabolic characteristics of fibre types in human skeletal muscle B Essén E Jansson J Henriksson A W Taylor and B Saltin Acta Physiol Scand 1975 95 153 165

III Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exe cise B Essén Submitted for publication in Acta Physiol Scand

IV Regulation of glycolysis in intermittent exercise in man B Essén and L Kaijser Submitted for publication in J Physiol

The papers will be refer ed to by their Roman numerals

I addition some hitherto unpublished results will be presented

Abbreviations ppearing I the text and figu es

AMP	= Adenosine monophosphat
ADP	= Adenosine diphosphate
ATP	= Adenosin triphosphate
CP	= Creatine phosphate
DHAP	= Dihydroxy acetone phosphate
FFA	= Free fatty acids
F 6-P	= Fructose 6-phosphat
F 1 6-P_2	Fructose 1 6-diphosphate
G-1 P	= Glucose 1 phosphate
G-6 P	Glucose-6 phosphate
GAP	= Glyceraldehyde 3 phosphate
Gl 1 P	= Glycerol 1-phosphat
HK	= Hexokinas
ICDH	= Isocitrat dehydrogenase
NAD	= Nicotinamid adenine dinucleotide
NADH	= Nicotinamide adenine dinucleotide (reduced form)
PDH	Pyruvat dehydrogena e
PFK	= Phosphofructokina e
P_1	= Inorganic pho phat
SDH	= Succinat dehydrogenase

Introduction

The proportion of energy production in exercising skeletal muscle derived from diff rent extra and intramuscular sources depends to a large extent on mode intensity and d r tion of wo k a well a on factors such a diet and physical work capacity (for ref see Gollnick 1977 Wahren 1977) The metabolic pathways f r the degradation of lipids nd ca bohydrates to release ene gy for muscle work i volves complex system of regulatory mecha isms (nervous hormonal and cell lar) These determine the interplay between lipid and carbohydrate utiliza- tion by affecting the r te limiting enzymatic teps thereby changing the rat through the pathways so that energy is released in rel tion to demand (for ref se Scrutton and Utt r 1968 Newsholme and Start 1973) Information on the regu- lation of metabolism ha to a great extent been obtained from a imal in vitro studies pref rentl lly on the rat heart V riou experimental conditions have been used such as hypoxia i chemi mechanical activity a well as perf sion studies with different ubst ates (for ref see Neely Rovetto and O an 1972) F thermore studies have been made i vitro on the properties of the regul tory enzymes Such studies have shown that carbohydrat utilization i ffected by int acell l r factors timul ting or inhibiting glycolysis t the hexokinas phosphorylas phosphofructokinase and py uvate dehydrogenase steps (Neely and Morgan 1974) Any proc s that consumes ATP in the cell g phy ical work will simultaneo ly in rea e the ADP AMP and P_i (Cai and Da i 1962) levels which a shown to timulate the PFK step (Passonneau and Lowry 1962) whereas increased level of ATP and CP on the othe hand inhibit this step (Mansour 1963, Krzanowski and Matschinsky 1969) Change in the $\frac{ADP}{ATP}$ ratio is then considered to a t s an important feedback signal regul ting the t of glycolysis and ATP formation F thermore a i crease i f tty acid availability suppresse carbohydrat uti lization by inhibition of glycolysi t the PFK step which is considered to be mediated by increased cit t l vels (Garla d Randle and Newsholme 1963 P r- meggiani and Bowman 1963 P sonneau and Lowry 1963) In addition it is shown that glycolysis i als inhibited by lowe ing f pH which affect the PFK and phosphoryl se steps (Danforth 1965) Thus changed level of subst ates and meta bolit as well s alte ed pH affect the rate f glycolysis

Skel tal muscle is composed f a mixture of fibres of diffe ent types having diff rent metabolic characte istics (Needham 1926) The red fib es have many mitochond ia and enzyme systems fo oxidative metabolism l rge blood supply and high myoglobin content indicating a high oxidative capacity Lipid content is the mai fuel and glycogen content i very low The white fibres on the othe hand have few mitochondri and enzyme systems fo lactat production small

amounts of myoglobin and a limited blood supply thus indicating a high glycolytic capacity Glycogen content is large and lipid stores small (Beatty and Boceck 1970) It has also been shown in the pigeon pectoralis muscles that white fibres are involved in brief bursts of intense activity whereas red fibres a e only involved in sustained activity (Parker and George 1975)

In several animals the proportion of each muscle consist predominately of one fibre type (Dubowitz and Pearse 1960) In man fibre types are mixed in most muscle groups (Edst öm and Nyström 1969 Gollnick Armstrong Sa bert IV Piehl and Saltin 1972) Thus in man these fibres must be studied separately for a more detailed analysis of metabolic regulation as whole muscles only give an overall picture It is then essential to relate the metabolic esponse to the degree of involvement of different fib e types in specific work situations

Work of such a high intensity that it can be sustained during only a short period of time is accompanied by a high rate of glycogen depletion and lactate accumulation and a great contribution of carbohydrate to oxidative metabolism (Pruett 1970 Saltin and Karlsson 1971) However when work of an equally high work load is performed as intermittent exercise in short bouts interrupted by short est periods it can be sustained for an extensive period of time and energy demand will fluctuate from a high to a low level between the wo k and rest pe iods (Åstrand Åst and Christensen and Hedman 1960) Metabolic esponse will be more simila to continuous moderate than to intense exercise with lower glycogen deple- tion smalle lactate accumul tion (Saltin and Essén 1971 Edgerton Essén Saltin and Si pson 1975) and significant utilization of lipids s suggested from espira to y exchange atio (R) (Ch istensen Hedman and Salti 1960) Consequently if i t rmittent and conti uous work of equal loads a e compa ed the metabolic e- pons is see to be different (fo ef see Salti Essén and Pederse 1976)

The diffe ence i subst te tilization between intermittent and continuous intense exe cise may be due either to diffe e t patte ns of fibre type acti a tion i the two situations or that factors egulating subst ate tilization a e brought i to play d ring the est pe iods of i termittent exe cise Among the varied possibilities fo eg lation the present study focuses pon the following hypothesis With each wo k period i i termitte t i tense exercise high en rgy phosphat s ar consumed and leads to stimulation of glycolysis and cit ic acid cycle acti ity whil i the est pe iods ATP and CP a e esynthesized and gly- colysis and citric a id cycl acti ity is eta ded Also in the ea ly phase of each rest period when cit ic acid cycle activity is still high the citrate le el will be low B t the mo cit ic acid cycle activity is retarded the more cit te will accumulate d e to an ssumed conti uous acetyl-CoA production f om fatty

acid oxidation The citr t will then penetrat the mitochondri l membrane and ffect t limiting steps of glycolysi thereby f rther retarding glycolysis in the beginning of the subsequent work period A compa ison of continuous and intermittent exerci e th s off rs opportunities for the analysi of mechanisms which cont ibut to the regulation of glycolysis in intact man

To test this hypothesis the present seri s of experiments were performed and the specific aims were

1 To tudy quantit tively intra and extramuscula carbohydrate and lipid uti-li ation in intermittent and continuous ex rcise

2 To study the glycogen depl tion patt rn in different fib types in connec tion with exercise and t evaluat whether r not specific activ tion pattern for muscle fibre involvement can explain differences in metabolic re ponse For thi purpose and f r studies of the metabolic charact ristics in diff rent fibre types methods wer developed f r qua titative analyses of sub trate levels and enzyme acti itie i ingle mu cl fibres in the human skeletal mus le

3 T analyse the metabolic chang d ing rest and wo k periods in intermittent intense exe cise i o de to lucidate the met bolic vents during rest pe iod which might be of impo tance f r the subst at utiliz tion in the subsequent work bout

Mat i l nd Method

Subjects

Altogethe i teen healthy men participated in tudies I III and IV They we e firemen policemen o tudent of which most studied physical training They ve aged 24 year (20-41) i ge 180 cm (174 193) in height 74 kg (62-87) in weight and 3 9 l/min (2 9-4 3) in maximal oxygen upt ke In tudy II thi ty-five healthy men and five healthy women aged 20-39 years with different occupa tional nd outine physical acti ity l els parti ipated

All subjects we informed of the nat re purpos and possible isks involved before gi ing their volunt ry consent to participat

The investigations were approved by the Ethical Committee at Karoli ka Insti tutet

Expe iment I procedure

All exercise tests we e pe formed in the pright position on an lectrodyna-mically loaded bicy l ergomet r (Siemens-Elema Stockholm Sweden) at 60 evolu tion pe minute Maximal oxygen uptake we determined ft 6 mi warm-up t e submaximal wo k load in preparatory test

In study I the contribution from ca bohydr tes and lipid to oxidative meta

bolism was studied in five subjects d ring 60 min intermittent intense exercise (15 sec work 15 sec rest) at a load that demands maximal oxygen uptake with continuous exercise (mean 299 W) and compared to 60 min of continuous exercise at a load (mean 157 W) selected to yield the same integrated oxygen uptake Thus approximately the same total amount of work was performed in the two types of exercise

Catheters we e inserted percutaneously into both femoral veins and the femoral artery on one side The tips of the femoral catheters were introduced approximately 8 cm in the proximal direction Blood samples were drawn simultaneously from artery and vein at different time intervals during the experiment for analyses of arterio-venous differences of substrates oxygen and carbon dioxide Leg blood flow was analysed at 15 and 50 min by analyses of arterio-venous differences of dye infused in the artery ^{14}C-oleic acid was infused between the 50th and the 60th min of work for estimation of fatty acid uptake Muscle biopsies from the vastus lateralis of m. quadriceps femo is were obtained at rest and after 5 and 60 min of work for analyses of intramuscular substrates

I st dy II muscle biopsies were taken at est f om vastus lateralis m soleus m gastrocnemius and m. deltoideus in order to study metabolic characteristics of the different fibre types in human muscle This study was intended to serve as backg ound to study III in which the glycogen depletion within the diff ent fibre types was analysed during 60 min intermittent exercise (15 sec wo k 15 sec est) at a simila load as in study I (299 W) 60 min conti uous exe cise of app oximately half that i tensity (157 W) and 4 to 5 ml conti uous exe cise at ma ical i tensity (266 W)

In st dy IV 60 mi intermittent exercise (15 sec work 15 sec r st) was pe f med t the same high wo k load as i study I and III (284 W) and compa ed to conti uous exercise of similar work load (280 W) to exha stion In o de to st dy metabolites which might have affected glycolysis in i termittent exercise muscle biopsies f om vast s lateralis were obtai ed both at the end of a wo k and a rest pe iod I conti uous exercise muscle biopsies we obtained at end of wo k and du i g the subseque t est period

An lytical methods

O ygen pt ke nd hea t t Expi ed ai was collected in Douglas bags and the volume meas d by a spirometer O_2 and CO_2 were analysed with the Haldane technique Hea t ate wa det rmined from electrocardiog aph ecordings

L g blood flow Leg blood flow was det rmined by constant infusion of indocyani e g en (Ca diog een ®) dye in the femo al artery as desc ibed by Jorfeldt and Wahren (1971)

Blood analyses Glucose was analysed in whole blood by the glucos oxidase reaction (Hugg tt and Nixon 1957) Pyruvat lact te glycerol and citrate were determined by deproteinizing 0 2 ml of whole blood in 1 ml ice-chilled 0 6 M perchloric acid and then e t alizing the sample with pota sium bicarbonate After centrifugation an liquot was taken f om the supernatant and nalysed by enzymatic fluorimetri methods as described by Lowry and Passonneau (1973) Free fatty acids in plasma wer analysed by ga liquid chromatography (Hagenf ldt 1966) Oxygen and carbon dioxide content in blood were analysed with the van Slyke technique (P ter and van Slyke 1932)

FFA turnove and regional exchange Albumi bond 1 ^{14}C-ol ic cid was infused continuously at consta t rate of approximat ly 0 4 μCi/min afte 50 min exercise and simultaneous a t ri l and venous samples were then t ken for the estimation of ^{14}C-olei acid pecific activity (Hagenfeldt and Wahren 1968) T rnove rate f oleic acid a well as log upt ke nd release of total FFA were calc lated as described by Wahren Hagenf ldt and Felig (1975)

Muscle biopsy technique Muscl biopsies were obtained using the percutaneous needle biopsy t chnique (Be gström 1962) The skin nd fascia 12 16 cm bove the patella on the l teral portion of the thigh were anaesthetized by xylocal or carbocain and an i cision was made through kin and f cia with scalpel The biopsy needle was inse ted 3 4 cm int the mus le nd ft withdrawal the mu cle sample was frozen immediat ly i liquid nitrogen and sto ed at 80 C until an lysed Not mo tha two samples were obt ined from the same inci ion

Comment on analyses of biopsy sampl s On of the maj r problems with the bi psy technique is that muscl biopsies du ing work are taken afte the muscl ha topped cont acting i at the best 3 6 sec after the end of work Changes occu ring i the mu cle du i g the few seconds lapsing from the end of muscle cont action to cutti g a mu l piece with the biopsy needl have not been directly st died i man Differences shown between immediately t end of work pe iod and 15 ec lat i e ting phases of i termittent exe cise re in all probability qualit tively co rect although the flu tuation occu ring i the muscl over the rest period a e quantitatively very hard to determi e

Changes may lso occ r d ri g the time laps between from t king to freezing of the muscle samples Thi wa tudied i a control experiment Two samples we obtained at rest and six sampl s aft r conti uou exercise to exha stion and the obtained muscle ample we di ided i to sever l part The fir t pa t was frozen withi 5-10 sec and the remai ing ft r f ther delay of up to 60 sec The res lts hown i fig re 1 illustrat that only light changes seem to occu fo most metabolit s and the v iations were withi the methodological ror

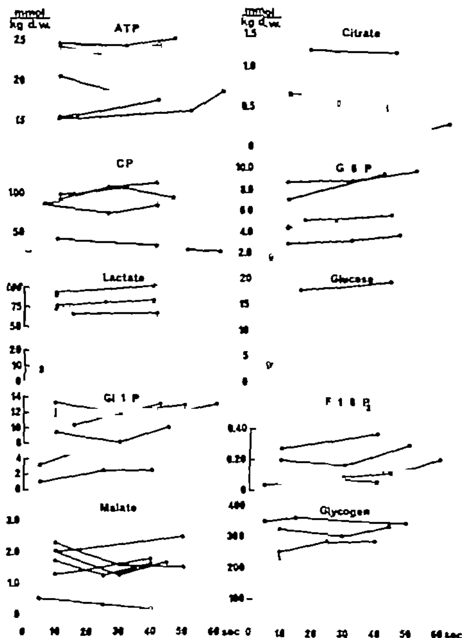


Figure 1 Content f ATP CP lact te Gl 1 P malate citrate G-6 P gl cose F-1 6-P_2 and glycogen in muscle frozen in liquid nitrogen at dife ent time inter als after the biopsy was obtained from the muscle
o = biopsies taken at rest • = biopsies taken ft exercise to exhaustion

Only lact te seems to increase slightly with time Thus the samples in the present study have not been the subject of any drastic changes during handling as they we e always frozen within seconds The above dat indicate that biopsy data qualitatively reflect the state of metabolism in the muscl tissue and the quan titative error is probably rather small

Muscl analys s The muscle samples were weighed at $20^{o}C$ and nalyses were performed ither (tudy I) on the wet tissue or on freeze-dried materi l (tudy II III and IV) Analysis on wet tissue is desirable when comparing concentra tions f soluble metabolites in mus le and *blood However the great admixt re* f blood adipose and connective tissue which sometime is found in a mu cle sample makes the dissected freeze-dried mate i l prefe able for compa ison of intramusc le metabolites

The freeze-dried material was weighed at constant tempe t re ($23^{o}C$) and humidity (35 %) on a Cahn electrobalance thereafter ext cted in ice-cooled 1 5 M perchloric acid and neutrali ed with 2 M pota sium bicarbonate

Aft r cent ifugation aliquots from the supernat nt were t ken for enzymati fluo imetric analysi of different intermedi tes (ATP CP G 6-P F 1-6-P_2 Gl 1 P malate itr t glucose lact te) of the metabolic pathway s de sc ibed by Lowry t l (1973) Glycogen we analysed s glucose re idues after the muscl sampl had been hydroly ed with 1 M HCl t $100^{o}C$ fo 2 hours (E sén and Henriksson 1974) Triglyce ides were analysed by extraction of neutral fats from the muscl sample with Folch extract The chloroform phas we retained and fter e poration hydrolysed and glycerol content was then determined (Che *ni k 1969*)

Preparation and cl s ification of fibr s

Freeze-d ied material was placed unde a dissection microscope (40-80 X) and f agments f i gl fib es were dissected out The fibres were then cut i to one *l rger pa t taken fo biochemical* analyses afte being weighed on a quartz fibre balance and smalle part wer t ken for cl ssification in typ I IIA and IIB fib es by staining fo myofibrill r-ATPase t pH 9 4 *aft r cid and lkaline pre*incubation Analyses of ATP CP and citrate we e performed on 50 150 µg pooled fib es of the same type

The methodological rror of blood and muscle analy es (n≥20 sample) exp es ed as the coefficient f variation (C V) of ingle value

(C V = SD of the difference between double values $\frac{1}{\sqrt{2}}$ $\frac{100}{\bar{x}}$)

Blood analyses Glucose 1 7 % lact te 3 6 % pyruvat 7 5 % FFA 2 % glyce rol 7 % O_2 content (sat 95 %) 0 7 % O_2 content (sat 35 %) 2 1 % CO_2 content 0 6 %

Muscle analyses (Two pieces from the same biopsy) Glycogen 6 3 % triglyceride 17 0 % lactate 4 2 % Gl 1 P 4 3 % malate 7 % ATP 4 5 % CP 5 7 % citrate 7 5 % glucose 6 1 % G 6 P 7 2 % F 1 6-P_2 8 2 %

Res lts

Oxygen uptake heart ate blood flow and respirato y exchange ratio (study I III IV)

Oxygen pt ke and heart rate with I termittent intense exercise (15 sec work 15 sec est) were similar to continuous exercise at half the work load and corresponded to 50 60 % $\dot{V}O_2$ max Continuous intense exe cise at similar high work load demanded 100 % $\dot{V}O_2$ max (Fig 2)

Arterial femor l venous (a fv) oxygen diffe ence in intermittent intense was 10 % higher than with continuous moderate exercise whereas the contrary was found for blood flow Thus the leg oxygen uptake calculated from a f oxygen differen ce and leg blood flow and the lung o ygen uptake were similar in the two situations Simila alues (0 87 0 90) for leg espiratory exchange ratio (RQ) and lung respiratory exchange tio (R) were also observed with intermittent intense and continuous moderate ex rcise

Blood borne ubst tes (study I) (Fig 2)

Arterial gl cose con entration did not change ignificantly during 60 min intermittent i te se o continuous mode ate exercise The glucose uptake was simila in the two ituations the lowest values being found in the early phase of xerci and the highest at end of the exercise pe iod

A te i l lactate concent ation increased to a simila magnitude i the early phas of continuou mode ate and i termittent i tense exercis but remained at the same le el throughout intermittent exercise whereas with continuous moderate ex rcise a prog ssive decrea e occu red A net elease of lactate was found during the whole period of i tense i termittent exerci e i contrast to conti nuous moderate exercise A similar pattern as fo lactate was see for pyruv te

No significa t diff ence in a te ial fatty acid concentration was found between the two modes of exercise and similar leg uptake nd release as well as total turnover rate was found in the two situations

Intramuscul s bstrate and metaboli t s (study I III IV) (Fig 3)

Glycogen depletion we simil in i termittent int se and continuous moderate exercise after both 5 and 60 min duration Significantly more marked depletion was found after 5 min of continuous i tense exercise than fte the corresponding period of intermittent exercise In five additional subjects in similar experi ments as in study I the t iglyce ide content was further inve tigated at rest

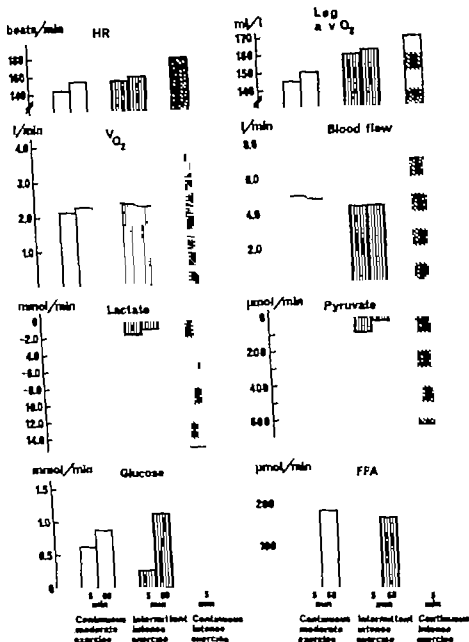
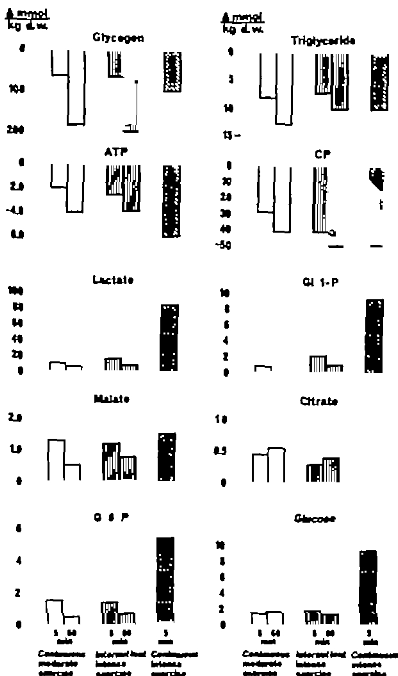


Fig re 2 V lues from study I III and IV on hea t rat oxygen uptake leg a v O_2 difference blood flow and uptake nd release of lactate pyruvat glucose and FFA at 5 and 60 mi of continuous moderate and i termittent i tense and 5 min con tinuous intense exercise to exhaustion Partly hatched ba s denot values obtained from literat re (Kaijse 1970 Wahren and Jorf ldt 1971) Work load in i ter mittent and continuous i tense exe cis is almost twice that in continuous mode r te exercise



Figu e 3 Values from study I III and IV on the Inc ease or decrease In glycogen triglyceride ATP CP lact te Gl 1-P malate citrate G 6-P and glucos fter 5 and 60 min conti uous moderate and Int rmittent Intense and 5 min continuous intense exercise to exha stion Work load in I termittent and continuous intense exercise is almost twice that in continuous moderate exe cise

and after 5 and 60 min of intermittent intense and conti uous moderate exercise (Table I) T iglyceride content had decreased after 5 min in i termittent s well as in conti uous moderate exercise A slight further decrease in triglyc ride content was found aft r 60 min of both int rmittent intense and conti uous moderate exercise

F r compa ison purpos s triglyceride content we analysed at rest and after exe ci e in thre of the s bjects that performed continuous intense exercise to exhaustion in tudy IV (Table I) All three subject showed a decreased value fter 5 min conti uous i tense exercise

F thermor in three additional subjects pe forming conti uou moderate exe ci e as in study I G 6-P glucose itrate lact te ATP a d CP were analysed after 5 and 60 min fo comparison with intermittent exe cis (Fig 3) Int muscular glucose G-6-P Gl 1 P and lact te content inc eased slightly and simi la ly aft work periods in intermittent intense and with moderate continuous ex r cise in cont st to continuous i tens exercise where significantly more ma ked inc ease was found Both ATP and CP content decreased fte work periods with int rmittent i tense and continuous moderate exercise nd more so with conti uou i ten exercise *Citrat on the other hand was i creased lightly with both* i t rmittent i tens and continuous mode ate xercise wherea with continuou i tens exerci no change from bas l level was found F 1-6-P_2 content was lightly

Tabl I Triglyce ide content (mmol/kg dry weight) i muscl at rest and after rious types of exerci e

	Conti uou mod rat exe is (mean 140 watt)					Int rmittent intense exer ise (mean 270 watt)				
Subject	1	2	3	4	5	1	2	3	4	5
Rest	35	35	32	41	33	51	34	32	49	39
5 min	22	28	28	44	18	29	32	21	47	35
60 mi	*20*	*26*	*23*	*30*	*18*	27	28	25	30	37

	Continuous inten e exercise (mean 300 watt)		
Subject	6	7	8
Rest	53	34	23
5 mi	40	20	20

increased with both intermittent and continuous intense exercise

Metabolite changes in rest periods i i t rmitt nt ex cise (study I IV)(Fig 4)

With intermittent intense exercise increased levels for ATP CP and citrate were found at end of a rest period compared to end of a work period G 6 P content tended to increase and F 1 6-P_2 content to decrease with each rest period and thus $\frac{G\ 6\ P}{F\ 1\ 6\ P_2}$ atio changed and was higher at the end than at the beginning of the r st pe iod Gl 1 P and lactate on the other hand both tended to decrease with each rest period

Metabolit changes in   cov ry in conti uous i tense exercise (study IV)(Fig 4)

ATP CP and citrate showed prog essive incre se also in the 3 min recovery phas after continuous intense exercise An increased $\frac{G\ 6\ P}{F\ 1\ 6-P_2}$ ratio was also found after 1 mi of ecovery compared to immediately after continuous exercise to exhaustion Lactate and Gl 1 P both increased slightly over the first 15 sec of recovery but we significa tly lower than this value after 3 min of recovery

S bstrat content   phosphagen level and enzyme acti ities i differe t fibre types (study II III)

The glycogen content was found to be only slightly but significantly higher i type II than type I fibres although large variation around the mean was found fo both type I and II fibres In contrast the intramuscul r triglyceride

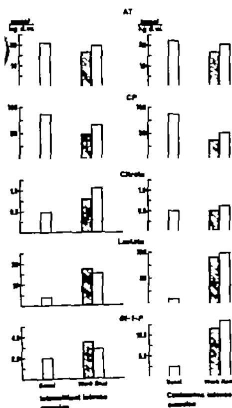


Fig re 4 ATP CP   it ate Gl 1 P d lactate content 1 at   st (basal) and at the e d of 15 sec work period and 15 sec rest period in i t rmittent ex rcise (data from 5 15 30 and 60 mi combined) 2 at r st immedi tely nd 15 20 se   fter 5 mi continuous inte se exer ise Equally high work load in both situations

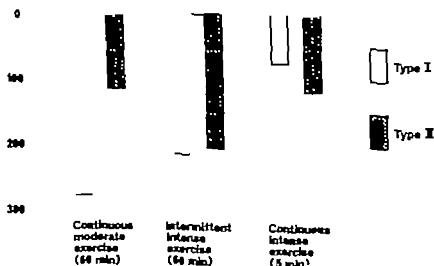
content was 1 5 2 times highe In type I fibres The oxidative potential of the fibre types as indicated by SDH activity was approximately 1 5 times higher In type I then type II fib s whereas the glycolytic potential as indicated by PFK acti ity was twice s high In type II

Glycogen tilization In diffe e t fibre types (tudy III)(Fig 5)

With continuous mode ate exercise a mo e pronounced depletion of glycogen occurred in type I than type II fibres whereas the opposite was found with con tinuous intense exercise I intermittent intens exe cise the depletion was similar in the two fibre types but small per unit time than with continuou intense exercise

ATP CP and cit te content I different fib types with conti uous inten e exe cise (Fig 6)

ATP CP and citrate were analysed on pooled fibr of ither type I or type II At rest the basal alue in both fib e types we simila as egard ATP b t for CP lway slightly higher in type II fibres A ma ked de reas had occurred for ATP and CP I both type I and II fibres t the end of continuous ex rcise to exha stion a d after 3 mi recovery an increa ed level was found Citrate content was I 11 sampl highe in type I than type II fibr s at rest well as lso du ing th whol period of recovery At end of exercise the cit t



Figu 5 Glycogen depl tion i type I and II fibre ft r 60 mi continuous mode at and I termittent intense exerci e and 5 min continuous i tense exer

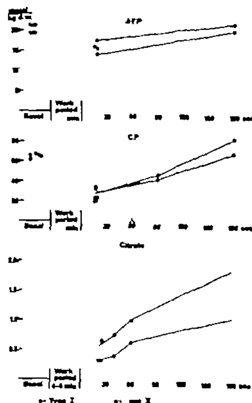


Fig re 6 ATP CP and citrate content I type I and II fibres at est (basal) a d t different time I t rv ls in recovery after continuous exercise to exhaustion

content was not diffe ent from basal but the eaft r showed a p ogressive increase in both fibre type

Di c ion

One of the basic findings f the p esent se ies of experiments is that t simila high wo k loads less glycogen is utilized nd lipid cont ibut mo to oxidative metabolism when exercise is performed i t rmittently (15 sec work 15 sec est) tha continuou ly The ove ll metaboli respon e to intermittent exercise is more simila to continuous exercise at about half the load than at an equally high work load Thi may be due either to factors which affect the *rate of the metabolic pathway o alt rnatively to diffe ent involvement of* fibre types In the following both possibilities will be discussed in detail Substrat utilization

Earlier studies on i termittent and continuous intense exercise have indi cated a different metabolic response as seen from blood lactate and respiratory exchange ratio (Åstrand t al 1960 Ch istensen et al 1960) That the met bolic response with continuous and intermittent intense exercise differed markedly at the same work load wa also confirmed in the present study (Figs 2 and 3) With continuous intense exercise there was a ma ked glycogen depletion and pyru-

ate was converted to lactate for the reoxidation of NADH to NAD Lactate production must have been even greater than suggested from the accumulation of lactate in the muscl since there also is a release from muscle The amount of lactate release in this situation was not measur d but has been calculat d from litera tu e data on arterial venous difference for lactate (Kaijser 1970) and blood flow (J f ldt t al 1971) and is included in Fig 2 The ma ked increase in Gl 1 P and mal t found with ontinuous intense exercise fu ther sugge ts that the conversion f dihydroxyacetonephosphate (DHAP) to Gl 1 P and oxalacetate to malate can be tilized as supplement ry pathways for reoxidation of NADH

The bove findings and lit ture data on glucose ptake a d integrated leg oxygen upt k uggest that the greatest part of the energy demand during continuous intens exer ise i covered by glycogen breakdown (Åstrand and Saltin 1961 Kaijser 1970)

Less glycogen was used pe unit time when i tense exercise was pe formed intermittently than when it was pe formed conti uously This glycogen saving effect seems t have been accompli hed both by hift towards more oxidative metabolism and by great cont ibution from other subst tes than glycogen notably lipids Glycogen and glucose tilization with l ctate end product generate 2 3 mol ATP whil complet oxidative deg dation yields about ~36 mol ATP per mole glucose The was l s ccumul tion per unit time of lactate s well as Gl 1-P du ing i termittent than d i g continuous intense exercise indicating that NADH reoxidation wa more readily ccompli hed by int amitochond i l oxidative met bolism (Fig 3) A g eate oxygen ilability due to reloading of myoglobin sto s s ha previously been sugg ted may have contrib ted to this (Åst and Åst and Ch isten en and Hedman 1960) I the pre ent st dy it was calcul t d that the myoglobi t es could have cont ibuted lmost half the xygen d ficit produced by each work period The other half was hown to be covered by phosphagen break down and la tate production which previously has been indicated to cont ibut to the energy release i i t rmittent exe cis (Edwa d Ekelund Har is Hesse H ltman Mal her and Wigertz 1973) In ddition to a more ffi ient tilization of glycogen d ring int rmittent exer i almost half of the oxidative met bolism wa cove ed by lipid ubst ates apparently from both intramuscul r t igly rid to es and fatty acid t ken up from the blood

The diff ence i metabolic esponse between i termittent nd conti uous i ten e exercise may then be due to eg l tory factors det rmi ing the int rpl y between lipid and carbohyd t tilization o selective activation of fibres with diff rent metaboli charact istics

Muscle fibre i vol ement

A selective glycogen depletion of muscle fibres has been shown to occur when the motor nerve is stimulated in the rat (Kugelberg and Edström 1968) Most studies on fibre involvement during exercise in intact man have used glycogen depletion as indication of fibre involvement (Gollnick Piehl and Saltin 1974 Gollnick Armstrong Saubert IV Sembrowich .hepherd a d Saltin 1973) Also in the present st dy the degree to which different fibres were involved in work was evaluated from glycogen depletion patterns However there a e many factors that limit the reliability of this method Other substrates than glycogen are a ailable to the muscle cell and can be utilized for energy productio such as glucose fatty acids and triglycerides These are all used at different rates depending on the work intensity the duration of exercise and their avail bility As discussed above a certain amount of glycogen tilization in a muscle fibre can correspond to very different energy productions depending on whether lactate production or oxidative metabolism predominates Furthermore glycogen resynthesis may occur in the muscle cell However with these limitations in mind glycogen depletion over a period of time must indicate that the depleted fibres have contributed to the tot l ten ion output and can th s be taken to reflect fibre recruitment Thus, predominantly type I fibres were recruited d ring continuous moderate exercise In contr t glycogen depletion after intermittent and intense continuous exercis occ rred in both type I and type II fibres in ather similar proportion and ifferences in fibre recruitment can thus not explain the divergent metabolic response between these two types of exerci e These findi gs also indicate that the maximal ten ion that the muscle has to exert i an import nt fa to influencing the activ tion of different types of fibres

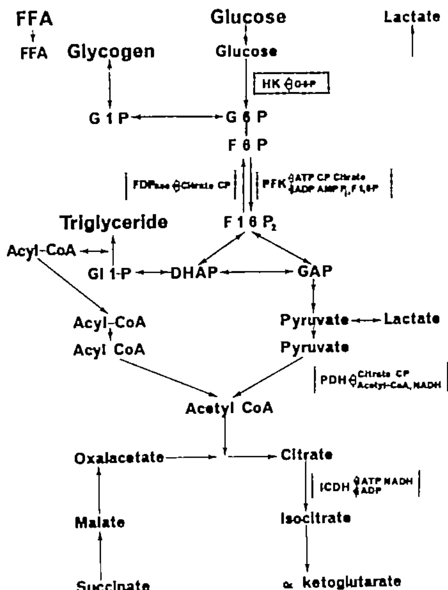
Fibre cha act ristics

In contrast to sever l a imal whe e great metabolic difference i found between red (type I) and whit (type II) fibres it is probably from the findings in the pr sent study that in man there is a less pronounced diffe ence in metabolic potential for lipid and ca bohydrate degradation between type I and II fibres Thus both fibre types have similar ATP CP and glycogen content nd although the lipid store was g eater in type I fibres there is lso a large amount in type II fibres The enzymatic profile shows that there is a relatively high oxidative as well as glycolytic capacity in both type I and II fibres Even though significant differenc s were found between fibre types type II fibres can in fact have equal high oxidative capacity as type I fibres especially in endurance trained s bjects (Jansson and Kaijser 1976) I addition the mean umber of

capillaries in contact with each fibre does not markedly differ (Andersen and Henriksson 1977) and a rather similar blood flow may thus supply both fibre types F ctors regul ting rate limiti g steps may therefore play a more important rol in determining the extent to which ither lipids or carbohydrat s re utilized in human fibres than in fibres of most animals

Regulatory facto s of subst ate tilization The regulatory scheme presented in Fig 7 has been adapted s a working hypothesis in the pr sent i vivo st dies of human muscle metabolism a d some of its implications have been tested sing intermittent exercise as an expe imental model The higher levels of ATP CP and citrate observed t the end of t periods as compared to work periods during int rmittent intense ex cise (Fig 4) indicate that these metabolites may be of importance for the reg l tion of ca bohydr te tilization s they all have been shown i i vitro studies to affect the ate-limiting teps in glycoly is Thus ATP inhibits the phosphorylase and the PFK step (Morgan nd Parmeggiani 1964 Ma sour et l 1963) and CP has lso been shown to i hibit the PFK tep (Storey and Hochachka 1974) The inhibition can be f lly eversed by ADP AMP and P_i (Newsholme t al 1973) Citrate inhibits both PFK nd PDH (Parmeggiani t al 1963 Passonneau et al 1963 Taylor and Halperin 1973) The effect of citrate i to potentiate the inhibitory effect of ATP on PFK (Newsholme et al 1973) The ATP inc ea at the end of rest pe iod i intermittent exercise will fu thermo raise the $\frac{ATP}{ADP}$ ratio nd this is known to decrease the activity f the isocit tedehydrogena t p (ICDH) in the itric acid cycle (Pl t 1970 Johnson and Han ford 1975)

The i hibition of ICDH i the presence of a continuous production of acetyl CoA from f tty a id oxidation could explain the inc eased it ate level ob erved t the end f est pe iod The extra oxaloacet te needed from the increase in itrat could have been gene ated by t ansamination f aspartat to oxaloacet te (Bowman 1966) or de ived from malate produced in glycolysis (Maizels Ruderman Goodman and Lau 1977) Thi cit te accumulation at the end of est pe iods will potenti te the inhibitory effect of ATP on PFK and thereby reta d glycoly is t the start of the next wo k period in i termittent exercise and thus facilit te fatty acid oxidation The increased $\frac{G\text{-}6\ P}{F\ 1\text{-}6\text{-}P_2}$ ratio at the end of each est pe iod *is a f th i dication of the i hibition f the PFK step* The i tramitochond i l ccumul tion of cit t cetyl-CoA and NADH generated i the β-oxidation f f tty acids could f rthermore i hibit PDH (Keely t l 1974) the eby f rthe limit ng the tilization of ca bohydrat The continuous slight release of lactate and py vate that occu red du ing the whole period f int rmittent exerci e may i dic t that pyruvat wa produced in excess of what could be oxidized to ac tyl-CoA



Figu 7 A schematic presentation of metabolic pathways I volved in degradation of lipids and carbohydrates Facto s timulati g I hibiting the rat limiting enzymatic steps re indicated by ⊕ o ⊖ re pectively

That there is a higher rate of glycolysis in continuous than in intermittent intense exercise is shown by the greater glycogen depletion This is also reflected by the levels of metabolites regulating the rate limiti g steps 1 ATP CP and citrate which Il were lower at end of work in continuous than i intermittent intense exercise Furthermore the increase in met bolite level in the period immediately after exercise showed different patt rn between int rmittent and continuous intense exercise (Fig 4) After a work bout in intermittent exercise citrate level increased rapidly wherea after continuous exercise the increase was more gradual As an effect glycolysis was not so much retarded during the first 30 sec of recovery a shown by the i creasing levels of Gl 1-P and lactate That the rate of glycoly is is only g adually re tarded in this ituation is further supported by the gradual ret rn of ATP and CP and the increase in citrat that occu red ove 180 sec of ecovery The progressive increase in $\frac{G-6-P}{F\ 1-6-P_2}$ ratio that occurred ove the first mi ute of recovery is an indication that the PFK tep is then influenced in this situation

The metabolites ATP CP and citrat which as suggested by the present study may et rd glycolysis were also analysed i type i nd II sepa tely fter exercise to exhaustion where glycogen depl tion data indicat recruitment of both fibre types (Fig 6) ATP and CP we e f und to be decreased in both type I nd II fibres and in the recovery similar increase occu red supporting tardation of glycoly is in both fibre types Cit ate levels were greater in type I fibres which probably is a reflection of the somewhat great oxidative capacity of thes fib es However the increase in citr te in recovery followed the same pattern suggesting that the itrate level is fa tor regul ting glycoly i and ca bohydrate tilization in both fibre type Thus it i likely that the glycogen-sa ing effect of ATP CP and citrate we present i both fibr types in intermittent exercise also both type I and II fibres were activated i thi situation

Citrate and CP ar not only inhibitors of PFK but lso activ t f muscl fructose-1 6-diphosphat s (Fu and Kemp 1973) Since fructose-1 6-diphosphata acti ity is present i human mus le (Krebs and Woodford 1965) the possibility exists that glycerol 1-phosphat i partly sed fo ynthesis f glycogen during each rest period in intermittent exercise However it is more probabl that Gl 1 P is f rthe degraded in the glycolytic pathway and oxidized used in the glycerol 1 phosphat shuttl since CP did never reach initi l level i rest periods

I tr ve su extramu cul subst t tili tion

The intramuscul r subst t to es in the f rm of glycogen nd t iglycer

decreased rapidly during the early phase of all types of exercise indicating that not only glycolysis but also lipolysis is activated rapidly by exercise To what extent intramuscular triglyceride breakdown may contribute to total energy release with continuous intense exercise has not been e aluated in the present study A progressively greater contribution from blood borne substrates was observed during both intermittent intense and continuous moderate exercise Thus glucose and FFA together contributed to over 30 % of the oxidative metabolism t end of exercise The utilization of blood borne substrates during continuous intense exercise has not been studied to any g eat extent due to technical difficulties The short duration of work and large ariations in blood flow and substrate levels limit the possibilities of quantitative meas rements However with intense forearm exercise a release rather tha pt ke of glucose was observed (Wahren 1970) The contribution of plasma FFA to oxidative metabolism has been shown to inc ease with time and dec ease with load (Ahlborg Felig Hagenfeldt Hendler and Wahren 1974) Consequently it was probably of minor import nce in the intense continuous exercise

The change i substrate utilization f om int amuscula glycogen to glucose taken up from the blood is probably related to the G 6 P level i the muscle i vitro studies have shown that G-6-P inhibits hexokina e acti ity and decreases intramuscul r glucose phosphorylation (Crane and Sols 1955 Newsholme d Randle 1964) During the i itial phase of exe cis G-6 P ccumul ted in mu cle in the present study and would thus have limited the phosphorylation of gl cos G 6 P content decreased and gluco uptake i reased during the subsequent exer ise period as shown by compa ison of the 5 and 60 min value for both intermittent and continuous mode te xerci e (Figs 2 and 3) The extremely high level of G-6-P ob erved aft continuou intense exe cise would suggest that glucose uptake from th blood is inhibit d The simultaneous i tramuscul r glucose accumulation found i this situation is most lik ly refl ction of th free glucose produ ed by glycogen breakdown (Field 1960) Thus the high int amuscular glucose level uggests that phosphorylation of gl cose was inhibited

Summary nd conclusions

1 With intermittent exercise at equally high work load as in continuous exercise a smaller glycogen utilization and Gl 1 P and lact te accumulation was found

2 The contribution from intra nd extracell l r s bstrat so rces to oxidative metabolism was imilar in i termittent (15 sec wo k 15 sec est) exercise t high work load and in continuous moderate exercis at half that load

3 The smaller glycogen utilization with intermittent than continuous exercise was in part due to greater oxidative met bolism and i part to a inc eased con t ibution from lipids

4 The small glycogen utilization in intermittent intens exercise was not due to a diff rent patt rn of fib e acti tion as both type I and II fibres showed glycogen breakdown which was lso found with continuous intens exer cise Thi lso indicate that work intensity i important for fibre activation

5 The met boli charact ri tics f type I and II fibres indicated that human fib es ha e capa ity for both carbohyd te and lipid degr dation and facto affecting met bolic pathway hould therefore be of importance in both fibre types

6 The increas in ATP CP and citrate levels during rest period in intermittent i tens ex rcis supports the hypothesis that these metabolites y ergi tically ffect t limiting st ps thus r t rding glycolysis t the st rt f each subsequent wo k period The mentioned fluctuations i the metabolites eem to occ i both type I and II fib es

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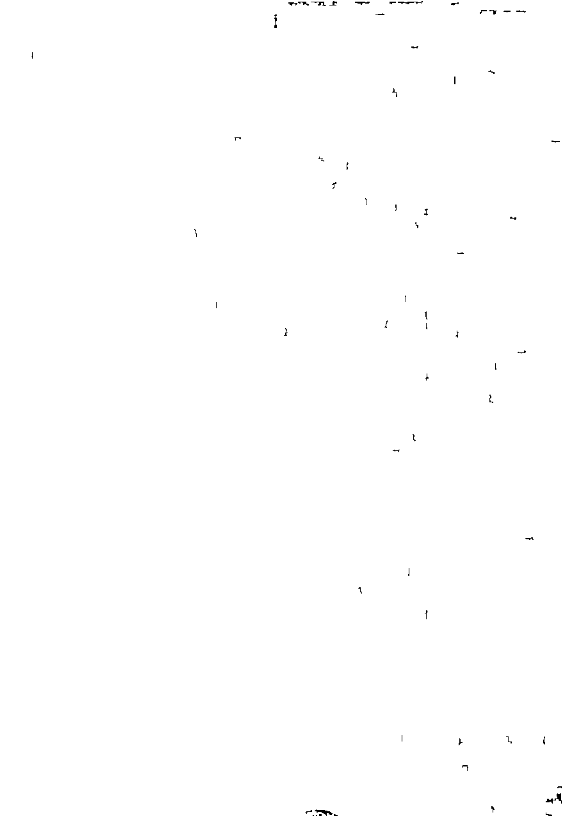
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Intracellular pH and Energy Metabolism in Skeletal Muscle of Man

With Special Reference to Exercise

By
Kent Sahlin

Stockholm 19/

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SUPPLEMENTUM 455

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I Sahlin K Harris R.C & Hultman E : Creatine kinase equilibrium and lactate content comp red with muscl pH in tis ue amples btained after isometric exerci Bio hemical Journal 152: 173-180 1975

II Sahlin K Harris R.C Nylind B & Hultman E : Lactate content and pH in muscl samples btained ft r dynamic exercise Pflügers Archiv 367: 143-149 1976

III Harri R C Sahlin K & Hultman E : Phosphagen and lactate contents of m quadricep femoris f man after exercise Journal of Applied Phy iology 43 852-857 1977

IV Sahlin K Palmskog G & Hultman E : Adenine nucleotide and IMP contents f the quadri eps mus le in man aft ex ci e Accepted f publi ation in Pflüg rs Archiv

V Sahlin K Harris R C & Hultman E : The creatine kinase reaction in human mus l during ecov ry from exerci e Submitted fo publication

VI Sahlin K Alvest and A Berg tröm J & Hultman E : Intr llular pH and bicarbonat concentration as det rmined in biopsy samples from the quadriceps muscl f man at rest Clinical Science and Molecular Medicine 53: 459-466 1977

VII Sahlin K Alvestrand A B andt R & Hultman E : Intr cellular pH and bi arbonate concentration in human muscl during r covery from exercise Ac epted f publication in Journal f Applied Phy iol gy

In addition some unpublished esult and calculations ar included

The pape s will be r ferred to by their Roman numer l

CONTENTS

ABBREVIATIONS AND DEFINITIONS

Apparent equilibrium constant	the equilibrium constant calculated from the concentr tions of substrates and product when these are not at infinite dilution
Cr	creatine
DMO	dimethyloxazolidiene-2 4-dione
Equilibrium	a condition where the change in fr e energy of a transf rmation is zero (g the rat of the f rward and backward direction f an enzymatic reaction is equal)
GDP	guanosine diphosphate
GMP	guanosine monopho phate
GTP	*guanosine triphosphat*
IMP	inosine monophosphate
Intramuscular pH	intracellular pH and total mus le pH
MAR_{CK}	mas -action ratio for the re tine kinase eaction i (Cr)(ATP)/(PCr)(ADP)
Mas -action ratio	the product/s bstrate rati f a rea tion calculated from the total tissue content of subst ates and products
MVC	maximum voluntary contraction force
PCr	pho phocreatine
pH	extr cellular pH
pH_i	int acell lar pH
sl	lyke mmol H^+ (1) ^{1} (pH) ^{1}
Steady-st t	a condition where the rat f f rmation f substance equal the r te f it disappearance
TAN	t tal denine nucleotide content (ATP ADP AMP)
T tal mus l pH	ave age pH of both the extra- and intr cellular compartment in a mus le sample
W_{max}	the maximum load subje t can sustain for 6 min bicycl exercise

Value re throughout this study expressed as Mean − SD

INTRODUCTION

The hydrogen ion has no electron hell and will therefore have a very small ionic radie This will provide H with unique prop r- ties such as an extremely high reactivity The concentr tion of hydrogen ions or more precisely the activity in the int l r of the cell is very low (about 10^{-7} M) Small change in the hyd gen ion activity will however d t the high reactivity exert a profound influen e on the met bolism

The hydrogen ion activity has fundame tal rol in det rmini g the dissociation f weak acid and bas Chang in intr c ll l r pH (pH_i) will there afor influen the at f biochemi l ea tion by eff cts on the enzyme itself as well as by change in the i i f rm of involved subst ates and product Because f the hydro- phobi nature f the cell membrane the permeability f r obstances in ionic f rm will gene ally be much lowe than f the unioni ub- tance Changes in pH will the f re influenc the di t ibution f weak acids and bases over the membrane Thi i of importance t the teady-state concentr tion of certain substanc in the ll as well t the rate of rt in transport processes ove the ll membrane involving weak cid o bases

In rd to keep pH_i within the limit which are compatibl with lif rigorous control i nece sary The hift of H and lectro- lytes between the xt and int cell lar compartments ar con trolled by permeability f t rs (low permeability f H and HCO_3^-) and by active (i ene gy-consuming) transport sy tems This enables the cell t keep an intrac llula milieu whi h is compl t ly different in pH and electrolyt composition from the extr c ll l r fluid Gener tion and removal f H within the ell i regul ted by ries f contr l mechanisms preventing exce sive hange in pH Some f these will be discus ed in the present the i

Measurement f pH in blood i one f the mo t impo tant li- nical analyses Obtained v lue will represent the H tivity in blood and in the extracell la fluid surrounding the c ll In many linical situations changes in blood pH rep esent intra-

cellular changes in the same di ctions The quantity of the chang in pH_i in the different cell compartments can however not be judged from extracellular measurements It has even been shown that under some circumstances changes in pH are not only quantitatively diff rent but can also occur in different directions within the extra- and intracellul r comp rtment (Adl r t al 1965 a & b) Direct determination f pH_i will therefore be a prerequisite for studies f intracellular acid base balance

A v riety of techniques has been used to measure or calculate pH_i in different types of tissues:

1 Measurements of the degree of disso i tion of weak acids or bases within the intracellular compartment Carbonic acid (Wall ce & Hastings 1942) and DMO (dimethyl xazolidi ne-2 4-di ne Waddell & Butler 1959) are the most frequently used substances Ni otine is an xampl of a weak base (Adler 1972)

2 Microelectrodes of different types fo direct measurements in the int ac llular compartment (for references see Cohen & Iles 1975)

3 Measurement of the H^+ activity in the ti sue water after di ruption of the cell membranes by homogenization or other t chniques (Furusawa & Kerridge 1927)

4 Use f coloured pH indi ator (Piontek & Harb t 1971)

5 Nuclear magn ti sonance (Dawson et al 1977)

6 Calcul tion f intrac llul r mass action rati where one of the participant sub tance is H (Si sjö et al 1972)

For more compl t survey and descripti n of the method and their appli ation in differ nt situations the eade i referred to the reviews a ailabl (Caldwell 1956 Robson t al 1968 Waddell & Bates 1969 Cohen & Iles 1975)

The vast amount f pe formed studies and the l g number f empl yed methods re not nly ymptoms f the importance of pH_i but lso f the difficulti s involved

Most previ us studie hav been imed t lucidat the influence on pH_i by K depl tion o by change in extrac llular pH (pH) nd/or by vari tion f PCO_2 Only a few tudies have been aimed t tudying the l tion betwe n pH_i and intermedi ry met bolism (Longmo e t l 1969 Adle 1970 Folbergrová et l 1972 Si sjö t al 1972)

In the present tudies muscl ti ue w inve tigated Repe ted muscle sampl s an asily be btained from human skelet l mus le by the needle biop y t hnique des ribed by Berg tröm (1962)

Skel tal muscl constitutes bout 40 % of the total body mas and measurements in thi tissue will the ef re provide information of the acid-bas homeostasis f an important part of the t tal cellular compartment

A unique prop rty f the mus le i the capacity to a ler t energy metabolism by changing from re t to exercise Intensive exercise with anaerobic en gy utili ti n will also change pH_i The magnitude of the changes i rel tively unknown but can according to the pre ent tudies be very larg and will exe t a profound influence on the cell metabolism

Meas rement f intracell lar pH in man have because of the methodol gi l difficulties been very sparse and no tudies have previously been pe f rmed rel ti g pH_i to hang in content f high-energy phosphates and lactat

It wa th ref considered to be f g eat importance t :

1 *Develop methods f int amuscula pH mea urement* itabl f linical and physiological research in man

2 Study the influence of intens muscular exer ise on pH_i and on the acid-base exchang with blood

3 Investigate the rel tionships betwee intramuscula pH and the contents f lactat and f high-en rgy pho phate ompounds

METHODS

General

Subjects The nature and purpose of the study were explained t ach subject before their voluntary consent was obtained Most f the subjects were between 20 and 35 years of age As a group they were not especially well trained though most of them regularly participated in some form of physical activity More detailed physical characteristic f the subjects participating in the diffe ent investig ti ns are found in paper I-VII

Muscl sample have been taken from the l ter l portion of the quadricep femori with the eedle biopsy technique des ribed by Be g trdm (1962) Loc l anesthetization and preliminary incision of the kin at the muscl biopsy site we e mad bef re start f exercis The sampl s were usually of between 20 and 50 mg wet wt

Exercise te ts Dynamic exercise was pe formed by bicy ling in the sitting position on an l ctrically braked rgometer (Siemens Elema AB St ckholm Sweden) t a continuous pedalling rat of 60 rpm The employed work load are expr ssed as per cent f the maximum l ad the s bj ct could sustain fo 6 min (W_{max}) W_{max} was determined f r e ch subject in a preceding s ssion (usually on we k bef r the expe iment) ac ording to the method of T rnvall (1963) It ha previously been shown by measurement of glycogen and high-ene gy phosphat s that the lat al portion of the quadri ceps femo i mus le i extensively used d ring bi y le exercise (Hultman 1967)

Isometri ont actions wer pe f rmed with the kne extensor chi fly the quadriceps femoris muscl on a chair d scribed by Tornvall (1963) The contra ti n f rce was ustained until p edet rmined l ad ould no long be maintained The empl yed ontr tion f ce is expre sed as p r ent f the maximum voluntary contraction forc which the subj t could ustain f r at least 2 s (MVC)

An lytical Methods

Met bolite content in mus le Muscl ampl s intended fo metabolite determinations wer frozen fast as pos ibl in the needle (3-6 sec delay during sampling) with liq id freon maintained t its freezing point (150 C) The samples wer f eeze-dried di sected free from connective tissue and blood and extra ted with $HClO_4$ (0 5 M) The neutralized extract was analyzed for metabolites by enzymatic method which are based on the diff enc in bsorption betwean NADH and NAD Detail f the procedu for di ection and ext action f samples and the analyses of 11 metabolite except inorganic phosphat (P_i) and inosin 5-monopho phate (IMP) have been d scribed by Harri t 1 (1974) P_i was analyzed enzymatically acco ding to Gawehn (1974) IMP was analyzed in the muscl ext a t with high-performanc liquid chromatography (f d tail se IV)

Blood sampl s Routine chemical analyses usi g an Autochemist sy tem (Aut Chem Instrument AB Lidingö Sweden) we e used for det rmination of prot in and electrolyte in plasma A id base va iable we e analyzed in an automatic ystem (Radiometer Copenhagen Denmark; type ABL1) with pH and PCO_2 l t od Lactat and pyruvat were analyzed in neutralized pe hl ri acid extracts by enzymatic method

Intramuscular pH homogenat technique Afte withdrawal f the needl from mus l the sampl s wer frozen as fast s po ible They were weighed fro en and homogeni d t 0 C with a sol tion (5 ml per g wet wt) cont ining 145 mM KCl and 10 mM NaCl (I) r 145 mM KCl 10 mM NaCl and 5 mM iodoaceti id IAA (II & V) The pH f the homogenate was me su ed t 38 C with a micro l trod equiring volume f about 20 µl The pH was f llowed with conne ted re rde and the value btai ed aft r ext apol tion to ro time was used By including IAA in th homogenat ol tion the continuous d reas in pH duri g the meas rement therwise btained was preve ted Influen e f IAA ddition on bt i ed v lues was investigated i two muscle ampl Ea h sampl we di ided int two part and analyzed f r muscl pH with the two diff rent homog nate sol tion The results (without IAA pH 6 97 7 04 and with IAA pH 6 98 7 03 r spe tiv ly) howed no diff renc

Int amuscul pH - CO_2/HCO_3^--method The mu l ampl were frozen as fast po ibl (about 5 se) in the needle with f eon at 150°C i liquid nit oge The needles we thereaft se led

with tape to prevent contamination with tmospheric CO_2 and stored in liquid nitrogen until further treatment The mus le content of acid-labile CO_2 (TCO_2) was liberated with sulphuric acid and absorbed in $Ba(OH)_2$ solution which afterwards was titrated with 2 5 mM HCl (for details see VI) The content of extracellular w t r (H_2O_e) in the muscle sample was derived from the tissue Cl content and the extrac llular conc ntr tion by assuming a passive distribution according to the membrane potential The ontent of intra llular w ter (H_2O_i) was calcul t d from the tissue content of pot ssium

Calculation of intrac llular bicarbonate concentration $(HCO_3^-)_i$ and pH_i ar based on the followi g as umptions hloride is passively distribut d ccording to the membrane potenti l; PCO_2 in the muscle ell eq ls that in femoral venous blood o i ft r exercise the mean of that in arteri l and femoral venous blood extr cellular bicarbonate con ent ation, $(HCO_3^-)_e$ equals (after correcti n for a Donnan factor and the plasma w ter content) that in femor l veno s blood o is ft r exercise the mean of that in art rial and femo l venous blood; no carbamino compound exist in muscle (Butle et l 1967a)

$(HCO_3^-)_i$ and pH_i have been c lculated from:

$$(HCO_3^-)_i = \frac{TCO_2 - H_2O \quad (HCO_3^-)_e - (H_2O_i \quad H_2O_e)\ PCO_2 \quad S_1}{H_2O_i}$$

$$pH_i \quad = pK_a \quad 1\ g\frac{(HCO_3)_i}{S_2 \quad PCO_2}$$

where S_1 and S_2 ar the solubility constants f r CO_2 in mus le and in the intrac llul water phase r spectively

F r a detailed de ription f the analytical t chnique the c l culations and a full di cus ion f th validity of the ssumpti ns see VI & VII

Ref rence base In the present serie of studies different ref rence base f expression of v lues have been used (f t f ea dry weight f mus le dry weight f musel wet weight of muscl and in some cases corre tion of values to constant total creatine content) A di cussi n f the inv lved problems can be found in III & IV For conve ion f value exp e sed per g dry muscle (the usual referenc bas for met bolites i the present studi s) to g wet wt values hould be multiplied by 0 23 (when the water content is normal i e 77 %)

INTRAMUSCULAR pH AND LACTATE CONTENT
AT REST AND AFTER EXERCISE

V lue f int amu l pH at rest I tr ll l pH in r ti g muscle was d t rmined usi g the CO_2/HCO_3 method t 7 00 - 0 06; n=13 (VI) T tal muscl pH in r ti g mus l was d t rmi ed with the homogenat t chnique to 7 080 0 034 (II) Homogenization f mus l will mi the int a and t acell l compartment and measured pH will thus be i fluenced by the volume buff r capacity and pH f the i t and extr ellul r fl id i the mus l sampl It can be cal lated that the ext ac llul fl id i the homogenat will i - ease th measured pH value i sample take at t by bout 0 03 unit (H_2O_i 89 % H_2O 11 % pH 7 4 i t c ll l buff apacity ext llular buffe c pacity)

As me tioned i the introduction pH_i has been meas red with l rg numbe f *technique I mo t animal studi pH_i betwee 7 1* and 6 8 has been btained i re ting muscl The re lt btained in the present studies f human mus l are thu i danc with mo t published inve tig tion

The i t i of the ll i when omp ed with the xt cell l spac neg tively harg d (88 mV) A p sive di tribution of H^+ according t the memb ane potenti l would t an t ac ll l pH f 7 4 sult i a pH_i f 6 0 The d vi tion from passi e di tribution f H does no it t an ti e (i en gy onsumi g) p ce f r extru ion f H (lt rnatively f inwa d t an po t of HCO_3) Some videnc exi t that thi p ocess i linked to the Na -K^+ pump which also mai t i the t ansmemb an potential and the i t llu- l le trolyte con ent tions (f r ref enc e Woodbury 1965)

Some controve y doe howeve till xi t in the tru value f i tracell l pH t st In some tudies with microelect ode a pH_i f 6 0 has been found by Cart r (1972) which would suppo t lectro-chemical equilib ium f r H The discr pancy i btained lues f pH_i me su ed usi g mi roel t ode (Ca t 1972) nd the t hniques ha been ugg sted to be due t struct r lly binding f wat r in the ell (Wiggi e 1972 Hannan & Wiggins 1976) If the wat in the ll i tru t lly bound t l g xtent

the activity coefficients for ions would be ppreciably different from that in the xtracellular fluid and the us d pK_a values when calculating pH_i from the dissociation of acid and bases would b in error and thus also the obtained values of pH_i It was however point d out by Robson et l (1968) that me red chang s in pH_i will not be aff cted as long as pK_a does not change

Butler t al (1967b) remarked that several glycolytic and oxid tive enzymes are almost inactive at pH 6 0 nd they questioned wheth r the muscle c ll could have any metabolic ctivity at this low pH The vast amount of studies with diff rent method f r pH determin tion which have shown greement in pH_i t rest and when chang s have been induced do also give onfide e in the pre ent studies and in the interpret tions given

Total muscle pH in rel tion to lactat ontent aft exercise When lact te is produced in muscl a corre ponding amount f H is released Total muscle pH decreased to 6 56 ~ 0 07 (n=8) after a fatiguing ontr ction at 68 % MVC (I) and to 6 60 $\pm$ 0 14 (n 9) aft r exhaustive bicycl exe cise at W_{max} (II) A linear elationhip was btained in these studies between accumul tion f lactate + pyruvat and decrease in total mus l pH (Figs 1 & 2)

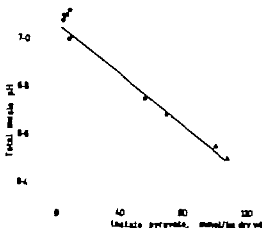


Fig 1 Relationship between total muscle pH and content f l tat pyruv te in mu cle sample taken at et (O) afte 15 min circul t ry occl ion (●) after iso metri co tra tion at 68 % of the maximum voluntary co t tion f e sustained for 25 se (■) o t fatigu (▲) F r d tails see I

pH = -0 00532 (lactate pyruvat) 7 06; r=0 96 n=24

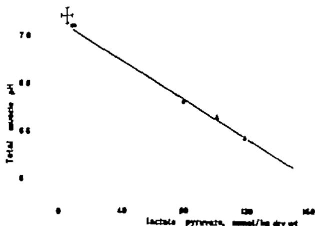


Fig 2 Rel tionship between total mu cl pH and nt nt f l tat pyruvat in muscle sampl s taken immediat ly after dynami exercise The mean − SD of t tal muscl pH and lactate content t rest (n=12) ar also shown The work was su tained f 5–11 min at a load f 50–75 % (●) and 100 % (▲ taken t exhaustion) f W_{max} F detail see II
pH −0 00413 (lact te + pyruvate) + 7 06; r=0 92 n=13

For given amount f lactat decrea in pH was mo e pronounced aft r isometri cont tion than ft dynami exer i The diff rence i probably due t the excheng of sub tances with blood which occu s during dynami exe cise but not during isomet ic ont action (see Chapt r 6) The highe amount of blood which gene ally is pr sent in muscl sampl taken ft dynamic exerci could also increase the i fluence f the extr ll l fluid on the measured pH v lue

Re overy The lact t content in mus le de ased expon ti lly with time after t rmination f exhaustive bicycl exercise (II) The initial lactat ontent was duced by 50 % fter 9 5 min recovery The initial t f lactat de rease was 7 7 mmol/mi per kg dry muscle The relationship between lactat pyruvat content and total muscl pH d ring recovery was gai line r (II) pH = −0 00521(lactat pyruv t) 7 22; r=0 93 27

Mu l samples have also been taken d ring the recovery phas f r measu ement of t tal content f acid labil CO_2 (TCO_2) and calculation f pH_i by the CO_2/HCO_3 method Mus l content f TCO_2 d cr ased from 9 84 − 1 39 (n 13) mmol/kg wet wt t re t to 4 64 − 0 76 (n=7)

1 min fter termination of exercise (VII) TCO_2 incr ased during rec very but was 20 min after termination f x rcis still significantly below the value t r t I t 11 1 r pH was calculated from TCO_2 muscl electrolyte and cid bas parameters f blood

The assumption underlying the calc l tions seemed justified f r values obtain d after 8 and 20 min recovery whereas lue after 1 min recovery *when the CO_2 content in muscle is low and* when f st flu tuatio s of CO_2 tension occur wer onsidered to be omewhat un ert in (VII) V l es of intramuscular pH at rest and during e overy from ha stive xer ise obtained by i g th homogenat t h ique (total mu cle pH) and th CO_2/HCO_3^- method (intrac ll lar pH) re compared in Fig 3

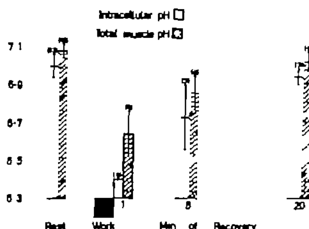


Fig 3 Compariso betwee tot l mu l pH and intrac llul pH i mus le sampl s taken t re t and d ring re very from xhaustive bi y l xe i The work w sust ined for 5 11 min Val e re given s mean SD numbers of obs rvation within brackets

Th data we bt ined i two diff re t studi but the omp ri n i justifi d by the imila ity in pe f rmed xercise and i the amount f l t te a cumul ted It ppears from Fig 3 that great chang s oc u in i t amus l pH d ri g xe ise b t that pH is re t r d aft 20 min ov ry T t l muscl pH i i 11 cases highe *than the rr spondi g i t* cellul pH As di cus ed bove thi s probably c equen e of the influen f extr llular fl id on th measu ement f t t l mus l pH The more centuated diff ren i me red pH betwe the two method ft 1 min covery co ld be d t the p viously mentioned uncert inty in v lue bt i ed by th CO_2/HCO_3^- method t this time to an

incr ased influence from th extracellular fluid in the measurements of t tal muscl pH Th d ta in Fig 3 do howev how that the two diff rent methods give eas nabl agreement as to the magnitude f hange and the time course d ring recovery

Relation f d t to lit atu e In study by Furusawa & Kerridge (1927) pH was analysed in homogenat s f t muscl It was found that pH d creased in l trically timulated heart muscl f om 7 07 at re t to 6 56 at fatigue and in gastrocnemi s mus le f om 7 04 at rest t 6 26 t f tigue The result from the p sent tudi s are in ag eement with th e finding

In studies by Dubuisson (1937 & 1939) pH on the surf f l trically stimulat d muscl wa meas red using glas lectrode From these experiments they aimed to gain inf rmation about the change in intr c llular pH The technique doe however uffer from evere limit tions measurement re aff ted by v ri tion i ti sue pol ization ext ac ll la onc tr tion f HCO_3 i diff ent from the intra ll l r concent tion and the respon is delayed by low activity f ca boni anhydras in mu cl The co ded chang will thus be diffic lt t i t rpret in t rms f pH_i chang

Inve tig tion of i t ratiti l pH in mus l have bown that t ansi nt initi l lkalinization followed by an increased acidification occur during cont action (Gebert & Sydney 1973 St inhagen t l 1976) The time ours of the pH hang s is in agreement with metaboli changes within the muscl (Danf th 1965) known t libe t ba (i e breakdown f PCr) and acid (i f rmation f l tic id) No quantitative inf rmation on int ac llul pH an however be obtained from these measurements i the ext a- ll l fluid

In tudi s by Piont k & He bst (1971) and He bst & Piont k (1972) pH_i ha been calcul ted from me su eme t of the bsorption f the pH indicat bromthymolblue which b d been locali ed int a- llularly The mean v lue f pH_i in resting frog mus l was 7 l During ont tion fast de re i pH occurred Th a tbo s do howeve not transl t the chang in pti l density to chang in pH and ont actions we not ust ined longe than 0 5 sec

In study by Rooth (1966) pH_i wa mea red by using the DMO method in the thigh muscl of t Only small decreas in pH_i from 6 64 t at to 6 57 aft r xhaustive running wa bserved In rder to btai reli bl v lue f pH_i with the DMO method

equilibration of the substance between the intra- and extracell lar compartments is necessary The equilibration time is considered to be as long as 1 hr (Waddell & Butler 1959) This condition was not met with in the tudy by Rooth (1966) and make interpr tation of obtained data difficult The same criticism co ld be raised to the study reported by Hermansen (1969) where ne subject was running intermittently for 20 min Intracellular pH (evaluated from DMO measurements) was found to decr ase from 6 88 in sting muscle t 6 73 after exercise

In another study by Hermansen & Osnes (1972) muscl ample were taken from m quadricep femoris after maximum bicycle exercise f short dur tion (1 2 min) Total muscl pH was measured with micro- lectrode in homogenates of the samples with similar technique as used in some of the present tudies (I II & V) The chang in muscl pH ob rved by Hermansen (decrease from 6 92 t re t t 6 41 afte exhaustive exercise) was imil t the l t r ob rv tion h epo t d

RELATION BETWEEN HIGH-ENERGY PHOSPHATE AND LACTATE CONTENT IN MUSCLE AFTER EXERCISE

Gene 1 con id r tion The content f high-en rgy phosphate in muscl aft r exerci e ha in this hapt been related to the content f lactate On bj ct f doing so i the previ usly de cribed los connection between l t te and intramuscular pH (Chapt 3) which mus t be on f the d t rminant f c ll met - bolism Anothe reason i that ti sue content of l t t will indicat the degree f anaerobic energy tilization and thus must bear some elationship tows ds the ther ana robic en rgy sou ces (i PCr and ATP) An advantag f relating high-energy pho phat co tent t lact te i the possibility f comparing xe ci f different type inten ity and duration and lso the po ibility of using dat as ref rence in linical e ea ch

ATP/ADP tio aft exercise Hydroly ti of ATP to ADP AMP p ovides the immedi t en rgy f most energy con uming pro se in the ll Ther have been numerous repo ts f de reased ATP and in reased ADP cont t in human mus l aft r exerci Though these lt r on i tent with the view f ATP utili tion duri g exe ise they re incompatibl with the known r t f ATP ynthe i in the cell

Resynthesi f ATP i extremely pid In f t t wa not until 1962 when Cain & Davi poisoned the muscl with 1 fluoro-2 4-dinitrobenzene that it could be proved that ATP wa the immedi t energy sourc in mus l Sampli g with the bi psy t chnique i the low and ther i del y f about 3-6 ec bef re ampl can be fro en It is theref e toni hing that d es ed ATP ontent an be bserved i mus l amples taken with the edl biopsy t chnique aft r xercise

In pape III a gen l rel tion between muscl ontent f lact t and ATP/ADP ti wa be rved aft xe i (Fig 4) Thi observation gg ts that the changes i ATP and ADP aft xe cise re more an adaption t an lt ed onditi in the ll nd an expr ssion f a new teady- tat rath than the ign of ATP tili

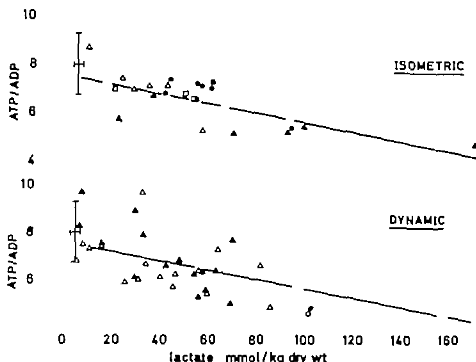


Fig 4 Comparison of ATP/ADP ft r exerci e with mus l la t t content Value at re t given as mean – SD (n=44) Uppe plot: value bt ined aft r isometri cont tion ustai ed at 40–45 % (△ ▲) 66 % (●) or 90–95 % MVC (□ ■) In one c e ont ti wa sustai ed to fatigue (▲ ● ■) and in thers not (△ □) Time to fatigue at 40–45 % MVC was 92 133 e at 66 % 35 63 and at 90–95 % 16 17 se
Lowe plot: values bt ined ft dynami exe i maintain d t 20–60 % (△ ▲) t 61 100 % W_{max} (○ ●) In some cases exer ise was sustained to exhaustion (▲ ●) and in the t (△ ○) Time t exhaustion at 20–60 % W_{max} was >30 min and t 61 100 % W_{max} 6 30 mi The relation hip between ATP/ADP and l t t hown by the continuous line in h subplot w lcul t d f om the tot l d ta obtained aft xe i
Fo det il e III
ATP/ADP 7 54 – 0 0196 (l t te); r=0 63 n 89

zation in the muscl Thi i further empha ized by the bserv ti that the d rea in ATP and the i re se i ADP pe i t f r at least 3 min if the ircul tion to the muscl is oc luded (I)

The r l tion between ATP/ADP rati and lact t ontent, indi at that an important facto regul ting the steady- t te could be intracellul pH.

Discussion of possibl me hanisms f regulation of the teady tat r ti f ATP/ADP T t l ATP and ADP r compo ed of diffe rent i nic specie including diff ent K- and Mg-complexes

$$ATPt\ tal = ATP^{4} \quad + HATP^{3} \quad + HMgATP \quad MgATP^{2-} \quad KATP^{3-} \qquad \text{Eq 1}$$

$$ADPtotal = ADP^{3-} \quad HADP^{2-} \quad HMgADP \quad MgADP^{-} \quad KADP^{2} \qquad \text{Eq 2}$$

Tabl 1 Dissociation constants for different complexes of ATP and ADP The constants for complexes involving K and Mg^{2+} are at 35 C and 0 16 ionic strength (Phillips et al 1966) whereas the constants for -complexes are at 25°C and 0 2 ioni strength (Melchior 1954)

				Dissociation constant (M)
$HATP^{3-}$	⟷	ATP^{4-}		$10^{-6\ 968}$
$HMgATP$	⟷	$HATP^{3-}$	Mg^{2+}	$10^{\ 2\ 80}$
$MgATP^{2-}$	⟷	ATP^{4-}	Mg^{2+}	$10^{-4\ 66}$
$KATP^{3-}$	⟷	ATP^{4-}		$10^{\ 1}$
$HADP^{2-}$	⟷	ADP^{3-}		$1^{-6\ 745}$
$HMgADP$	⟷	$HADP^{2}$	Mg^{2+}	$10^{\ 1\ 95}$
$MgADP$	⟷	ADP^{3-}	Mg^{2+}	$10^{\ 3\ 42}$
$KADP^{2-}$	⟷	ADP^{3-}	K	$10^{-0\ 68}$

From the data in Table 1 and Eq 1 & 2 the f llowi g equations an be derived:

$$(MgATP^{2-}) \quad \frac{(ATPt\ tal)}{1 \quad \frac{10^{-4\ 66}}{(Mg^{2}\ )}\left(1+ \frac{(H\ )}{10^{-6\ 968}} \quad \frac{(H^{+})}{10^{-6\ 968}} \quad \frac{(Mg^{2+})}{10^{\ 2\ 80}} + \frac{(K\ )}{10^{-1}}\right)} \qquad \text{Eq 3}$$

$$(MgADP^{-}) = \frac{(ADPt\ tal)}{1 \quad \frac{10^{-3\ 42}}{(Mg^{2}\ )}\left(1 \quad \frac{(H\ )}{10^{-6\ 745}} \quad \frac{(H\ )}{10^{-6\ 745}} \quad \frac{(Mg^{2}\ )}{10^{-1\ 95}} \quad \frac{(K\ )}{10^{-0\ 68}}\right)} \qquad \text{Eq 4}$$

where concentr tions are in mol/l and $(Mg^{2}\ )$ denot s the free concentration of Mg^{2+} From Eqs 3 & 4 and the dat in T ble 1 the concent tions of the othe f rms of ATP and ADP can be calculated

The concent tion f $MgATP^{2-}$ and $MgADP^{-}$ e in Figs 5 & 6 shown t decreas when pH or/and fre $(Mg^{2}\ )$ decreases

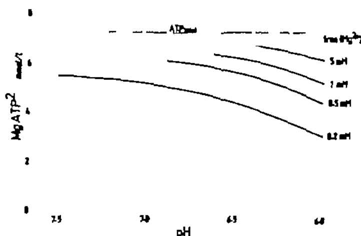


Fig 5 Concentration of $MgATP^{2-}$ at diff rent pH and concentrations of Mg^{2} T tal amount f ATP (7 27 mmol/l H_2O ~ 24 0 mmol/kg dry wt) and concent ation f K^{+} (160 mmol/l H_2O) are constant

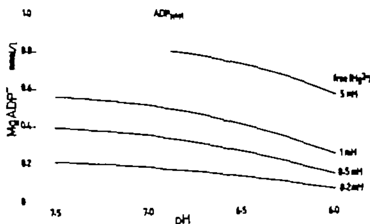


Fig 6 Concentr tion of MgADP at diff rent pH and con entrations of Mg^{2+} Total amount of ADP (0 970 mmol/l H_2O ~ 3 2 mmol/kg dry wt) and concentration of K^{+} (160 mmol/l H_2O) are constant

ATP binds Mg^{2} strong r than what ADP does and the proportional change in MgADP when pH decreases will the efo e be mor pronounced than for $MgATP^{2}$ Thi is al appa ent from the change in the $MgATP^{2-}$/MgADP tio (Fig 7)

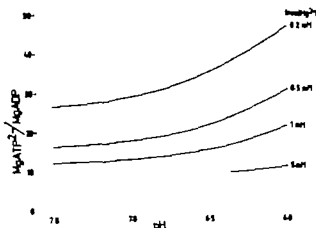


Fig 7 The rati $MgATP^{2}$ /MgADP t diff rent pH and centration f Mg^{2} T t l amount f ATP (7 27 mmol/1) ADP (0 970 mmol/1) and concent tion of K (160 mmol/1 H_2O) re constant

The intrac llular concentration f f ee Mg^{2} has been timated to about 1 mM (V 1 so t 1 1973) The sults in Fig 5 6 & 7 how that the concent tions f $MgATP^{2-}$ and $MgADP^{-}$ in thi regi n f (Mg^{2}) i sensitive toward chang in both (Mg^{2}) and pH

Many f the enzymes reacting with ATP and ADP behave as if $MgATP^{2}$ and $MgADP^{-}$ were the true ubst tes: hexokinase (Melchi & Melchior 1958) pyruvate kinase (Melchio 1954) phosphoglyce te kinase (Larsson Rasnikiewi z 1967) phosphofruct kinase (PFK) (Lowry & Passonneau 1966) adenyl t kinase (Ros 1968) creatine kinase (Watts 1973) The bs rved d creas i the ATP/ADP ti aft exhaustive exe cise (Fig 4) could be a phy i l gic l respons to the ffect f pH on the Mg-complexes f ATP and ADP

The flux through the reaction cataly ed by PFK is sually considered to be te-limiting fo glycoly is PFK i inhibited by H and the enzyme i lmost compl tely ina tive t pH f 6 4 (Danf rth 1965) This pH inhibition can partly be overcome by d rease in ATP and a cor esponding increase i ADP and AMP (Trivedi & Danforth 1966) The observed decrease in the ATP/ADP ati t high lactate content and thus low pH_i could the f re be metabolic gulation i d to keep the nece sary flux th ough the glycoly is

It has been shown that the ATP/ADP ratio within the mitochondrion is lower than in the cytosol (Elbers t al 1974) Th bserved decrease in the ATP/ADP ratio in muscle samples obtained after exercise could be due to changes in the ATP/ADP ratio within the mitochondrion at low oxygen tensions and at l vated concentrations of extramitochondrial P_i

Phosphocre tine ontent in muscl The content of phosphocrea tine (PCr) in muscle is very sensitive to changes in the metabolic situation It was first shown by H ltman et al (1967) and l ter confirmed in other inve tigations that PCr was utili od during exercise and decreased down to zero at exhaustion It was also shown that PCr during continuous work at a submaximum work load reached a steady-state level In paper III general relation between PCr and lactate content was bserved in muscle samples taken immediat ly after termination of exercise (Fig 8)

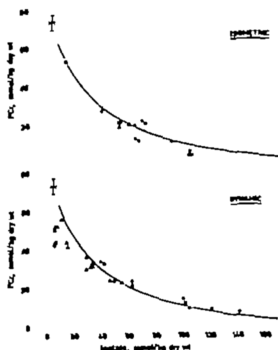


Fig 8 Comparison between PCr and lactate content in mu l Values at r st ar given a mean - SD (n=44) The continuous line shown in both bplot was derived from the total data aft r ex rcise Symbol as in Fig 4 Fo detail see III
PC = 34 e$^{-0\ 06(l\ ctate)}$ 51 6 e$^{-0\ 015(lact\ t\ )}$

The relation seemed to exi t irrespectively of the type inten sity and duration f the preceding exe cise When the irculation to the muscl was occluded no esynthe i f PCr ccurred (I: Table 1 V: Table 1 Harris et 1 1976) The dat indicat that PCr i at a teady-state level aft r exercise and that thi level is well related to the content f la tat When the blood flow in mus 1 is intact a rapid r synthesis of PCr occur It wa shown by Harris et 1 (1976) that the esynthesis fter exhaustive bi ycle exercise could be described by an exponential function with fast ($t_{1/2}$ 21 sec) and a slow ($t_{1/2}$ >170 sec) component The fast component corresponded t 82 % and the slow component to 18 % of PCr being resynthesized

The rel tionship between PCr and lact te in muscle during r covery from exhau tive exercise is as ppears from Fig 9 compl t ly diff rent from the lation obtained immedi tely after t rmination f exercis As the relation between l t te and t tal muscl pH i simil in these ituations it f llows that PCr content aft r exe ci i not exclusively linked to total muscle pH

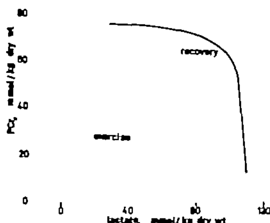


Fig 9 Comparison between PC and l ctate in muscl during covery from exhaustive bicycle exe ise Wo k times were about 8 min The continuous lin was de i ed by combining the time course of lactat disappearance du- ing recovery (II): lactate = 5 + 105 $^{-0\ 0733t}$ with the time course f PCr esynthesis (Harri t 1 1976) PC 75 - 60 e $^{1\ 98t}$ 13 e$^{-0\ 24t}$ (t denote min f ecovery)

The dotted line is taken from Fig 8

The influence of oxygen on the resynthesis of PCr was investigated by incubating muscle samples taken after a fatiguing isometric contr ction in atmospheres of oxygen and nitrogen respectively (V: Tabl 2) During 15 min incubation in oxygen PCr was resynthe ized from a starting value of 4 % to 68 % of the normal value at rest No resynthesis was observed when parallel muscle samples were incubated fo the ame time in nitrogen The experiment stre sed th importan of oxygen for the resynthesi of PCr and it was sugg sted that the availability f oxygen is limiting for the initial f st phase of PCr resynthesis The subsequent slow phase wa considered t be limited by the covery in intrac llular pH by influen e on the creatine kinase eaction (Chapter 5)

N chmansohn (1928) has shown that phosphagens can partially be synthesi d after a 5 sec tetanic contraction under ana robic conditi ns The pres nt results (I & V) where no anaerobic re synthesis f PCr could be det cted ppear to be in conflict with this study A po ible explanati n is that a non-steady-stat condition exist d in the study by Nachmansohn wherea in the present studies a steady tate in the creatine kinase r action was eached before the samples were frozen due to the delay in the sampling proc dure The differen e in contraction time might l be f impo tan

T t l adenin nucleotide ontent in muscl The size f the t t l adenin nucleotid pool (TAN = ATP + ADP AMP) shows in normal man at rest only mino int a- and interindividual variations (Harri t al 1974) A decreas f TAN has however been bs rved und some xtreme physi l gical conditions such as prolonged ischemi (Threlfall & St n 1957 Imai t l 1964) and t tani cont acti n (Canfi ld & Maréchal 1973) Decreased TAN con tent has al been bserved in pathological conditions involving mus l uch as h umat id arthritis (Nordemar et al 1974) and in severely ill patients uff ring from ircul tory and respiratory insuffici ncy (Bergström t al 1976)

In muscl sampl s taken from the same leg bef re and ft t rminati n of exercise an absolut decrease of TAN by abo t 15 % occurr d when the inten ity of the preceding exe cise was high re ulti g in a high content f l tat (IV) Breakdown f TAN in kelet l mus l occurs by d amination of AMP t ino in 5-mono phosphate (IMP) Analysi of IMP howed that this b tanc ac u mulated in mus l in an amount corresponding to the decrea e in

TAN (Δ IMP 2-6 mmol/kg dry wt) After 30 min recovery the basal content of TAN was restored and IMP had disappe ed from the muscle

The ccumulation f IMP in muscl aft r intense exercis i probably due to ctivation of AMP-d amina by a ombined ffe t f decreased intrac llular pH (pH ptimum for AMP-deaminase is 6 1-6 5 Setlow & Lowenstein 1967) and increased content of ADP and AMP (Fig 10) Deamination f AMP i sentially irr ve ibl and IMP can either be degraded and excr ted as hypoxanthine u at r lt rnatively by epa ate ro t be transf rmed back to AMP (Fig 10)

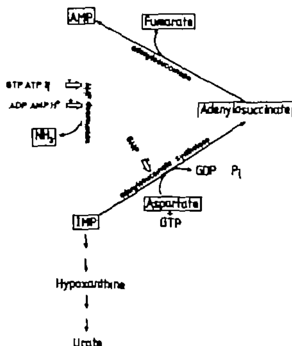


Fig 10 The purin nucleotid cy l and it regulation (⇒ inhibition; ⇒ tivation)

Thi cycl has been lled the purine nucleotid cy l (Lowen st in 1972) The phy iological importance of the purine ucleotide cycl i

1 Regul tion f the AMP level and se ndarily (by the adenyl te kinase rea tion) the ADP and ATP concentr tions in a way which will keep the energy cha ge (Atkinson 1968) at high level

2 Production of NH_3 which to l g extent will be combined

with glutamate forming glutamine which is known t accumulate in mus le during xercise (Bergström et al unpublished data) Accumulation of glutamine will partly buffer the increase in H associated with exercise (Chapter 6)

3 Production of carbon skeleton from amino acids to comb stion in the citric acid cycle

THE MASS-ACTION RATIO OF THE CREATINE KINASE REACTION AFTER EXERCISE AND DURING RECOVERY

C eatine kinase i believed t cat ly the f llowing eaction (Mihei et l 1961 Kuby & Woltmann 1962 W tts 1973)



The participation of H in the eatine kina reaction has two important phy iol gical co equen e

1 B eakdown f PC will abso b H^+ i ns Thi will d ri g the first second f maximum contr ti when PCr b eakdown i expe ted to occu without f rmation f lact t (Danf th 1965) in as the int ac ll lar pH This alkalinix tion will f ilitat the activation f PFK and the glycogen pho phorylase sy tem (Danforth 1965) and cc l t f rmation of l t t During sustained cont action when high amount f lact t i cumul ted depl ti f PC will be an important buff ring proces (Chapt 6)

2 If the reatine kina action is t equilibrium change in int ll l pH will ff t the content f PC and reatin

If the sy tem i implified and it is assumed that the creatine kinas reaction is t equilibrium and t t l ti sue tent f the involved metabolit are p opo tional t the concent ti t the enzymati it the following f rmul an be derived:

$$1 g\frac{(C\ )(ATP)}{(PC\ )(ADP)} = \alpha - \beta\ pH \qquad \text{Eq 5}$$

where α and β re onstant ($\beta \leq 1$)

U ing β 1 an excellent greement has been found in b in ti sue between changes in pH calcul ted from the reatine kinase ma acti ti (MAR_{CK} = (C)(ATP)/(PCr)(ADP)) and pH_i measured with the CO_2/HCO_3 t chnique (MacMillan & Si jö 1972 Si jö et l 1972) In the present tudies (I III & V) the relevanc f the equation has been investigated in muscl aft exercise

Muscl samples taken bef re and aft an isometri cont action unde teady-stat c nditions (i e local irculation occluded t least 1 min bef re mus le biopsy) we analyzed f tot l muscl pH and f the constituent f the cr tine kina reaction (I) A linear rel tionship was found between tot l mus l pH and

log(MAR_{CK}) Fig 11) The same r lation seemed to exist in all muscle samples taken immedi tely fter termin tion f exer ise (III) indicating that th creatine kinase reaction was t a steady state condition or at equilibrium

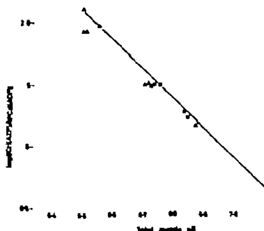


Fig 11 Relationship betwe n log(MAR_{CK}) i log((Cr)(ATP)/(PCr)(ADP)) and total muscl pH in mus 1 samples obtained at rest (○) fte 15 min circulat ry occlusion (●) and after isometric contr ction t 68 % MVC ustained fo 25 se (□ ■) to fatigue (△ ▲) □ and △ denote values where 20–25 sec mus 1 blood flow was allowed afte ntra tion Blood upply was occluded 1 min before biopsy
Fo d tails see I
1 g(MAR_{CK}) = 17 6 – 2 38 pH; r=0 92 n=34

It was postulated that the decrea in PCr bserved fter exer- ise was the con equence f an altered equilib ium state f the reatine kinase rea tion largely aff ted by changed intracellul pH (I) The value of β in these tudie (cf Eq 5) wa however 2 38 which exc ed the exp ted value i $\beta \leq 1$ When the recovery processes in muscl afte xe cis wer in estigated a deviation from the previous lationship was bserved (Fig 12) Thi was first believed to be the consequence of non steady -state condition during the early p rt of ecovery Experiment with occluded circul tion 1 min p i r to muscl biopsy did however show that the creatine kinase reaction was at a teady- -state r equilibrium during the whol re ov ry p riod (V)

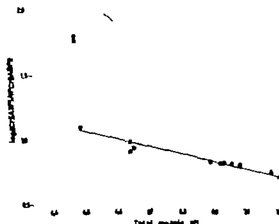


Fig 12 Rel tionship between tot 1 muscl pH and 1 g(MAR_{CK}) f log((C)(ATP)/(PCr)(ADP)) in muscle sample obtained t t (▲) aft r 0 1 (■) 1 (□) 4 (●) and 10 min (○) recov ry (with intact mus le blood flow) from exhaustive bicycl exerci e Blood upply was oc luded 1 min bef re mus le biopsy The regressi n line was calcul ted from v lues btained t re t and during 1 10 min overy
Fo detail ee V
log(MAR_{CK}) = 4 97 0 60 pH; r=0 85 n=24
The dashed line is the p evious rel tionship as ob t ined in Fig 11

In paper V it was hown that the av ilability of oxygen was responsibl f r the difference in MAR_{CK} between sampl s taken immediately aft termination f exe ci (Fig 11) and sampl s taken during the recovery period (Fig 12)

Discussion f the quilib ium hypothesi The fraction of ATP and ADP which are in the f rms f $MgATP^{2}$ and $MgADP^{-}$ respe tively have been calcul ted from Eq 3 & 4 (Chapt 4) t pH 7 0 (Mg^{2}) 1 mM and (K) 160 mM $MgATP^{2}$ = 0 92 ATP $MgADP^{-}$ = 0 53 ADP If it is assumed that the cre tine kinase reaction is clos to equilibrium and that there is no II 1 compa tmentalization - the ppa rent equilibrium constant f r the creatine kinase reaction K_{CK} (Cr)($MgATP^{2}$)/(PCr)($MgADP^{-}$)(H) can be calculated from the total tissue ontent f the involved metabolites K_{CK} ha in T ble 2 been calculated to 8 4 10^{7} 12 1 10^{7} and 22 0 10^{7} f r

skeletal muscl heart muscle and brain respectively Taken into account that bout 56 % of the ADP in skeletal muscl has been found to be bound to proteins (Seraydarian et al 1962) which increase K_{CK} in this tissue to about 19 1 values between different tissues are very similar

[illegible] Calculation of apparent equi[illegible] constant (K_{CK}) for the creat[illegible] [illegible] reaction ([illegible])

Tissue	PCr	Cr	ATP	ADP	AMP	$\frac{ATP}{ADP}$	$\frac{MgATP^{2-}}{MgADP}$ a)	K_{CK} × 10[7] (M^{-1}) b)
Skeletal muscle µmol/g dry wt [illegible] quadriceps of [illegible] (Harri[illegible] et al)	75.3 ± [illegible]	49.0 ± [illegible]	24 ± [illegible]	2 ±0.3	0.1 ±0.05		13.	[illegible]
Heart muscl µmol/g dry wt perfused rat heart 35°C ([illegible] 1966)	30 ± [illegible]	39 ± [illegible]	24 ± [illegible]	2.6 ± [illegible]	0.		14.[illegible]	12
Brain µmol/ wet wt [illegible] (Fries[illegible]) et [illegible] Bergstrom et al [illegible]	[illegible] ±0.05	5. ±[illegible]	3.0[illegible] ±0.04	0.2[illegible] ±0.004	0.0[illegible] ±0.001	1	30.	22
K_{CK} determined in vitro ([illegible] [illegible] 1963)								36 ±[illegible]

) $MgATP^{2-}$ [illegible] ATP [illegible] MgADP [illegible] ADP were calculated [illegible]

K_{CK} = $\frac{(Cr)(MgATP^{2-})}{(PCr)(MgADP)(\ )}$ calculated at pH [illegible]

The same finding has also been reported f r the mass-acti n ratio of the reatine kinase eacti n (MAR_{CK}) when different tis ues from diff rent pe ies were in e tigated (Bei & Newsholme 1975) The small v riation in the mass-action atio was here consid red to s ggest an equilib ium situation The exp riment lly d termined v lue f r K_{CK} (T bl 2) is howev r 15 30 time great than that c lcul ted in tissues and i thus in conflict with the q ilibrium hypothesi

The chang which cc rs in th MAR_{CK} ft r intensive exercise and the following ecovery pe iod doe lso seem to be in confli t with the equilibrium hypothesi The obs rved change in intra musc l r pH and the involvment of H in the r atine kinase equi librium an a shown abov not be the nly explanation of the chang s in MAR_{CK} in association with exercise

A theory b sed on the eq ilibrium hypothe is fir t advanced by Carlson & Sig   (1951) and lat r dopt d by Canfi ld & Maréchal (1973) and McGilvery (1973) i that 90 % of the ADP content in muscle is protein bound and not available in the reatine kinase reaction The small hang whi h is bserved in ADP aft r exercise would ac ordi g to this theory have a fa gre te influence on the

position of the creatine kinase equilibrium than when consid ring the total tissue content The mas a tion rati of the adenylate kinase reaction (MAR_{AK}) is however 0 2 0 4 (I III) and thus simil r to the apparent equilibrium constant for thi reaction (K_{AK} = 0 4 – 1 0 t (Mg^{2+}) 0 – 5 mM; Rose 1968) and 90 % protein binding of ADP is by this reason unlikely The lmost unchanged value f MAR_{AK} in muscl when great changes occur in the adenine nucleotide contents (Cain & Davi 1962) i ls in conflict with such a high deg ee of p otein binding

From the discus ion above it is lear that many factors influence the MAR_{CK} in a complex way It cannot be excluded that the creatine kinas rea tion i t eq ilibrium and a combined effect f availability f metabolite and changes in pH and (Mg^{2+}) t the enzymatic ite explain the observed changes in MAR_{CK} during exe cis and the f llowing recovery period

Dis us ion f the teady-stat hypothe i Creatine kina exi t in diff rent isoenzymes: muscl p cifi (MM) b ain specific (BB) and an int rmediat f rm (MB) The mu cl spe ifi f rm (MM) i dominant in k l tal mus l (Dawson & Fine 1967) Apart from these maj r f rms eatine kinas exist in a mitochondrial f rm (CK_{mit}) which eems to be localized on the outside f the inne memb ane (Jacobus & Lehning r 1973) The activity of CK_{mit} is higher in ti ues with highe robi capacity and is about 5 % f t tal activity in quadri p mus l f r t (Jacobs t l 1964 Murone & Og t 1973) It was postul ted by Jac bs t al (1964) and f the discussed by Be aman & Fouyo (1966) and Jac bus & Lehning (1973) that CK_{mit} functioned as mediato f ATP from the mitochondri t the cytoplasma The reatine kinase e ction wa thought t be t a teady tat whe e the backward reaction (PCr resynthe is) occurred t the mitochond ial memb ane in s ociation with oxid tive phosphoryl tion and the f rward eaction outsid mitochondria In isolated mitchond ia from heart ATP-ADP-t an locase and CK_{mit} appear to function as multienzyme ggregate which only lowly reacts with ext rnally added ATP but apidly with ATP formed in the mitochond i (Saks et l 1975)

By the t ady- tat hypothesis any f t whi h ff ct the sta-ef dne r both directions of the c atine kinase ea tion will chang the mass- tion atio A l ck of oxygen would p event resynthe is f PC and would thus expl i the finding in the pre nt tudi (I III & V) A dec ase i pH_i wo ld inc ea

the rate of the forward reaction (PCr breakdown) as this reaction has a pH optimum of about 5 0 (Nihei et al 1961) and decrease the rate of the backward reaction (PCr resynthesis) as this reaction catalyzed by CK_{mit} has a pH optimum of 8 0 (Jacobus & Lehninger 1973) A decrease in pH_i would thus decrease the steady-state level of PCr The influence of pH on the PCr level and the MAR_{CK} which has been observed in muscle tissue (Meyerhof & Lohmann 1928 Kammermeier & Rudroff 1972) would according to this hypothesis be explained by opp site influen on the reaction rate of the different directions of the creatin kinase reaction The observed changes of MAR_{CK} in the present studie (I III & V) would partly be due to influence of pH_i on the creatine kinas reaction and partly due to lack of oxygen

Chapter 6

BUFFER CAPACITY IN MUSCLE AND ACID-BASE EXCHANGE WITH BLOOD

Intr llul r buff r p ity The buffer capacity or buff value (β) of a soluti n was defined by van Slyke (1922) a the amount of free H r OH⁻ to be added in order t produce chang f pH of 1 unit:

$$\beta - \frac{\Delta H}{\Delta pH}$$

It wa sugge ted by Woodbury (1965) that the unit f β when measured in mmol(1)$^{-1}$(pH)$^{-1}$ should be designed Slyke (Sl) Thi t rm will be used in the f llowing

In addition to the physico-chemical buffe capa ity which only is dependent upon the amount f weak cids and bas and their *dissociation constant the ll has the buff mechanisms The* intracellular buff r cap city has by Siesjö & Mess t r (1971) been *divided into*

1 Phy i o-chemical buff ring
2 *Consumption r production f nonvol til acids*
3 Transmemb ane fluxe f H f HCO_3^-

The intracellul buff *capacity in skel tal muscl* be been determined in vitro by tit ation f mu l homogenates with H /OH⁻ *to* 40-65 Sl (Furusaw & Kerridge 1927) 62 82 Sl (Bate-Smith 1938) 48 59 Sl (Eck l et l 1959) and by v ri tion f PCO_2 in mu le homogenat t 53 Sl (Hei le & Piiper 1971) *The e value differ* from the buff r cap ity in intact mus l prep tions (about 15 Sl) as d t rmined by vari tion f PCO_2 (*fo r f* rences se Hei l & Piipe 1971) It ha been hown that the di epancy i due t transmemb ane flux e f H /and HCO_3 i intact muscl (*Heisl r &* Piipe 1972) A t iki g diff ence i here p esent between various ti ue During seve respi t ry acidosi H^+ app ar to be transported into the k l tal muscl ll but out from the ll i heart muscle and b in (Clancy & Brown 1966 Siesjö & Me set r 1971 Lai t l 1973) Thi mode f acid base regul tion seems to tilize the buff i g capa ity f blood and skel tal muscl f the protection of ther more itic l tissue

Evidenc exists that the in vivo buff r capacity i dep ndent upon the type (metabolic or r spiratoric) nd d gree of th acid--base disturbanc (Adler et al 1965 a & b Burnell 1968)

Comparison between Δ (lactat pyruv t)/Δ pH nd i t cellula buff r capacity Measurements of int amusc lar pressure and the change in muscle temperature (Edwards et 1 1972) have shown that the circulation within the muscl is arrested when the force of an isometric contr tion exceeds 20 % MVC (maximum voluntary contraction force) In the present tudies whe MVC >40 % the mus le thus xists in an isol ted condition and can be treated a closed sy tem Th amount f l ct te which i a cumulated d ring the ontraction will thus b c sponded by an equal amount f H^+ The ratio Δ (la tate + pyruv te)/Δ pH has afto isometric contr ction (I) been determined t abo t 57 mmol(1 H_4O) $^{1}(pH)^{-1}$ which is in the same range as the in vitro d termined buff r capacity f r muscle (40-82 S1)

Duri g bicy le ex rci the mus le i an pen system whi h ex changes substances with blood The atio -Δ (la tat + pyruvate)/ Δ pH after 5-10 min bi ycl exe cise w s det rmined t 73 mmol (1 $H_2O)^{-1}(pH)^{-1}$(III) I t a ellula bica bonate concent ti n decreas s from 10 2 mmol/1 H_2O at rest to about 3 mmol/1 H_2O aft r exha tive bicy le exercis (VII) and rr ponding efflux f H as carbonic a id will occur from muscl (i e 12 mmol(1 $H_2O)^{1}$ $(pH)^{-1}$) This amount is also t the same as the diff ren in the Δ (lactate pyruv te)/Δ pH atio between i omet ic and dynami exercise During dynamic exerci part of the lactate p oduced within the mus le will be transpo ted to the blood From the cal culations above it ppears as if this l ctat is accompanied by an equimolar amount of H Bas defi it and l ctate content in arterial blood do also show an equal increase d ing exe is (Fig 13) and do th refore suppo t this hypothesis The exa t mechanism fo l ctic a id transpo t ov the ll membrane i not fully understood I some re ent inve tigations it has been hown that t l ast p rt of the la ti acid is t ansported in undissociated form (Mainwood & Worsley-Brown 1975 Roos 1975) whi h implies that chang s in intr c llul and extracellular pH might be f importance to the t ansport proce

It has been shown that PCr ontent in muscl after exhaustive dynamic exe ci e is estored from 16 % to about 90 % f its value at rest d ring the first min of recovery (Harris et al 1976)

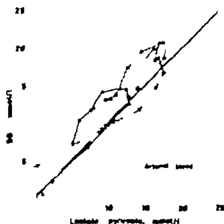


Fig 13 Base d fi it (BD) compared with content f lactat + pyruvat in blood from a t ri *brachialis* i 3 bjects Sample we taken t rest (△) duri g bicycle exer-ise (○) fter 0–2 min covery (●) and aft 7 30 min ecovery (▲) Subjects worked 5 min t 100 W and thereafter at W_{max} (275–300 W) until exhau tion Tot l wo k time wa 10–11 min and max p lse r t 170–190 V lues f om the same subje t ar j ined Content f pyruvat was t all time very small (<0 5 mmol/1) (Sahlin Alvestrand Brandt & Hultman unpubli hed d ta)

Thi means that 55 mmol PCr/kg dry wt formed from Cr and P_i PCr and P_i have pK_a value f 4 5 and 6 8 resp tively and it can be lcul ted that t an i t llular pH after exe cise of 6 4 about 22 mmol H /kg dry wt should be f rmed d ring the fir t min of rec very due t r yntbe i f PCr No further d eas in mus l pH do howeve cur during thi period (II) and it was sugg ted that H was t ansported out from mus l in exce f lactat during the early pa t f recovery Measurements of base defi it and lact t in rt rial blood support thi hypothe i Base deficit in se during the fi st min t s f recovery where-no rresponding change occu in l t t + pyruv te (Fig 13)

The mechanism behind the vents an only be speculated abo t The dri ing f s for passive movement f ions va the cell memb an is det rmined by the diff renc in lectro- hemi l poten-ti l f the ions i the two compartments The int ior f the muscle ll is neg tive (88 mV) compared with the ext ac ll l r

compartment and a p ssive distribution of H^+ over the cell mem-brane at pH_e 7 4 would mean a pH_i of 6 0 The true averag v lue of pH_i t rest is however 7 0 and activ (i energy consuming) transport of H^+ out of the cell is consid red to compens t f r the passive influx After exhaustive exe cise pH of rterial blood de-creases to 7 2 and pH_i down to about 6 4 Though the diffe en e in electro-chemical potenti l has decre s d influx f H i still energ tically favoured at a normal membrane potential If the mem-b ane potenti l decr ases afte exerci e t -50 mV it an be cal-culated that the hydrog n ion is in lectro-chemical equilibrium ove the ll membrane A further decrease i membran pot ntial or lternativ ly a further d c ease in pH_i as would be th ase if the esynthesi of PCr after exhaustive exercis oc urred with-ut a tran port f H^+ ver the cell membrane would change the conditions making a passive fflux of H^+ possibl It eems quit po sible that at the extreme onditions prevailing in exhausted muscl th membrane potential is de reased Meas rement of the membran pot ntial in mus le aft r ci culatory occlusi n for 3 hr hav shown that in thi situation it is linea ly related to con-tent f la t t and de rease down t bout 50 mV (E ger 1978)

Int a llular buff ing process s The contribution f diffe-rent sub tances to the intrac llular buffering towards l cti cid during dynami exe cise has been calculated in T ble 3 Th ub-stances have been divided int two groups The first g oup (physico-chemical buff ring) compris s the buffering whi h oc urs in the cell merely a consequenc f as oci tion of H with b se whereas the second group comp i es the buffering which occurs as a conseq en of enzymatic activity Th buffering of th first group i independent of the nature f the pH chang (at a constant PCO_2) whereas the buffering of the sec nd group i dependent upon the metabolic regulation and al ulated values consequently only valid unde the prev iling onditions In addition to the acid base changes d s ribed in Table 3 an accu-mulation f citrat and mal t an ccur under some conditions It ha however been shown by E sén (1978) that nly minor changes ccur during exhaustive bicycl exercise

Estimation f the buff ring by intrac llul r proteins varie (15 Sl Bate-Smith 1938; 17 37 Sl Woodbury 1965) and makes c lcu-l tion of total intra llular buff r capa ity un ertain to the same extent The estimat d intr cellula buff capacity f 68 Sl

Tabl 3. Contribution of different intracell lar transformations the buff ring towards lactic acid during exhaustive dynami exercise when in acellul pH decreases from 7 0 t 6 4

			ΔH mmol/l H_2O_i	Buff value (βl)	Footnot
Physico-chemic l buff ring (ati)					
HCO_3 H	⟶	H_2CO_3 ⟶ CO_2	7 2	12 0	
HPO_4^{2-} H	⟶	H_2PO_4	-4 3	7 2	b
ATP	⟶	HATP	-0.38	1 0	
ADP	⟶	HADP	-0.2	0 4	
Histid ne	⟶	Hhistidine	-0.07	0.1	d
Carnos ne (hi idine)	⟶	Carnosine (Hhistid ne)	1 19	2.0	
Protein	⟶	Hprot I		about 15	f
Produc ion consumption of acid or bases (dynamic)					
PCr	⟶	Cr P	15 4	25 7	g
ATP	⟶	IMP P_i NH_3	0 32	~0	h
Glutamat NH_3	⟶	Glutamine	-4 7	7 8	i
Glucose	⟶	Glucose 6-P	1 4	2 3	j
Glycerol P_i	⟶	α-Glycerol-P	8	-0.3	k
				Total About 60	

) Calculated from the change in intracell lar HCO_3 during exercise (VI)

b) Calcul ted from he P_i concentra ion at rest 13 2 mmol/l H_2O_i (IV) and pK =6 8 for H_2PO_4

) The change in he concentration of HATP (=HATP^{3-}+HMgATP) and HADP (=HADP^{2-}+HMgADP) was calculated from Eqs 3 4 and data from Tabl 1 (Chapt 4) assuming (Mg^{2+}) 1 mM and ( )=160 mM.

d) The change in he concentr ation of Hhis as was lculated from he h stidine concentration at re 0 38 mmol/l (Berg trõm et l 1978) and pK =6 0

) The change i the concentration of Hcarnosine wa alculated by us ng the carnosine content at rest 6 15 mmol/l H_2O (Berg rom et l 1978) and pK =6.0

f) Calculated from the histidine conten f muscle prot ins by Woodbury (1965) Ti ation of muscl ext act wi h and without prot ins gave 17 27 Bl for he prot buffer capacity (Bu e-Smith 1938)

g) Calculated from the obtained change in PCr af er exhaustive bicycl exercise (ΔPCr=63 µmol/g dry wt) and pK'=6 8 f H_2PO

h) Ca culated from the amoun accumulated IMP after exercise (3.7 µmol/g dry wt IV) and the final compos tion at pH 6 4 of ATP (HATP^{3-}+HMgATP 0 11 ATP_{total}) IMP (pK =6 23 for HIMP) and P (pK'=6 8 for H_2PO_4) Format on of NH_4 i inc uded i he g utamate-glutamine transformation

i) Ca culated from he change in acellul g utamine concent ation (18 9 mmol/l at est and 23 6 mmol/l f 10 min dynami exercise Bergström et l unpublished data) The calculated buffer value maximum f gure as some glutamine must have been exported to he blood.

) About 11 µmol lucose 6-P i accumulated per g dry muscl fte 2 min maximum bicycl exercise (Harr et l 1971) Calculations based on pK =6 1 for glucose 6-P and pK'=6 8 for H_2PO

k) About 8 µmol α-g ycerol-P accumulated per ry muscl f er about 10 min maximum bicycl exercise (unpub ished observat ons Ca culations based on pK =6.66 for α-glycerol 1-P and pK =6.8 for H_2PO

is however very similar to the ratio -Δ (lact te pyruvate/ Δ pH 73 mmol (l H_2O)$^{-1}$ (pH)$^{-1}$ as obtained from meas rements in muscle samples taken after bicycl exercis (II) and is lso in the same range as the experimentally determined buffer c pacity in vitro (40-82 61)

Chapt 7

GENERAL DISCUSSION

Mus le biop y t chnique The needl biop y t hnique is well suited for studi f th teady- t t composition f mus 1 Th f st changes which probably occur in the high-en gy phosphat and pH_i during short maximum contr tion can howeve not be de- t ted Thi i because f the d lay f 3-6 se d ring sampling bef re ampl s an be frozen It i ident that chemical rea tions will hang the compo iti f mus 1 tows ds a t dy- t t con- dition d ing the d lay In this the i I hav t ied t di cus the changes in high en rgy phosphate ompounds and lact te I mu 1 aft r exe cise in t rms f f t i fluen ing the t ady-stat Comparison between th pr sent data and those btained in animal expe iments whe e the mus 1 ha bee pidly fro en whil till in a tat f co traction should be made with aution It i probabl though that the absolut diff ren between the compo- ition of mus 1 at the instant f exer i t rmination and that measured in fro en biopsy sampl re quit small pe i lly i the cas of dynamic exe is

Studi f human skel tal mus 1 have some d antages ove animal expe iment The load typ and d tion f the p f rmed exe cise an be d t rmined bef the experiment Repeated sampl can be b- t ined from the same mus 1 and will implify tudi f hang and time co rs Th btained d t on mus 1 metabolism in normal ubj ts ca lso be tili ed as f enc in lini 1 i ve ti gations

Het og ity f mu 1 amples In normal man the quad icep mus 1 on i t f app ximat ly equal propo tion f fast twitch (FT) and low twit h (ST) fibers (Goll ick t 1 1972) with dif f re t enzymati (E &n t 1 1975) and co t til char t i ti A diff rent ne gy met bolism with diff rent rate f lact t pro- duction i xpe ted within the two fiber type Thu in animal ex pe iment it ha been eport d that l t t cumul tion oc p ima ily i FT mu l fibers (Baldwi & Tipton (1972) Analyse in sepa t d fibers from the same sampl taken ft xe i have

however shown that the l ct te content is not very different in the FT and ST fiber of the quadriceps muscle of man (Essén & Häggmark 1975 Tesch & Karlsson 1977) In the quadriceps muscle the fiber types are intermingled and diffusion of l ctic acid between neighbouring fibers might occur Essential f r the present studies is that if a transport of lactate between fibers occur thi i companied by the hydrogen ion Results from paper II and Fig 13 indicat that thi i the as

The intracellular compartment is not a homogen phase but consists of differ nt rganelles surrounded by membr ne It i possible that these differ as to pH and concentr tion f metabo lites Local variation of pH can al occu at the surface of prot in molecules and memb an s as a consequenc of ionic interactions Measurements of intracellular pH and co centr ti n of metabolit s will give some sort of a erage pH in the cell which might be diff rent f om the true value at the enzymatic site Heter genity f pH within the cell has been exten iv ly di cussed in lit rature (fo references see Cohen & Iles 1975) It is g nerally agr ed that mo t of the theo ti al bj tio s against absolut pH_i values dis ppear when change in pH_i ar tudi d It eems reasonabl that th different compartment ha connection and that a chang in pH_i i refl ted in the whol ll The cytosol make up the majo pa t f a mus l sample and me d absol t values f pH and met bolit s will p ima ily repr sent this compa tment

Impo tance of pH_i f the regulati f en gy met boli m The immedi te energy source fo muscular contraction i ATP The co tent of ATP is howe limited and would only last fo a few econd if the muscl wo ks at it maximum apacity ATP must th ref be resynthesized at abo t the same t e it i tiliz d ATP can be re ynthesiz d by breakdown of PCr th ugh glycolysi and by oxid tive pho phorylation The relative o cent tions of ATP ADP and AMP are the main reg l to s f the r t f ATP generation (Atkinson 1968 Newsholme & Sta t 1974) and do ls seem to be imp rtant for det rmining which sub t te to be used (i PCr glycogen glucose fat; McGilvery 1973) Mu l tissue has a high glycolytic ap city and can chang it gly olyti rat about 100 times At the maximum glycolytic r te lacti acid is accumulat d within the muscle c ll in high amount and will de ase intrac ll la pH To avoid destruction f acid labile ell omponents la ti a id pro-

duction must be limited It was already in 1955 con luded by Hill from experiments with elect ical stimul tion f muscle that fo mation of lactic acid ceas d at an int ellul pH of about 6 3 It has been shown that phosphofruct kinase (PFK) which is considered t be the r t limiti g enzyme f r glycolysi is inhibited in vitr at low pH (Danf rth 1965 Ui 1966) At the ATP co cent ation in r sting muscle (bout 7 mmol/1) PFK is lmo t compl tely inactive at a pH f 6 4 whi h i clos to the meas d pH_i aft exhau tive exerci (I II & VI) Eviden exi t that at leas t part f the pH inhibition i due t the a cumul tion f $HATP^{3-}$ which i a very potent inhibito f PFK (Lowry & Passoneau 1966)

Glycogen phosphorylase i ctivated d ring muscul contra tion An active phosphoryl se will produce hexose-phosphate which if PFK is inhibited would ccumul t in the muscl F rmation f hexose-phosphat from glyc gen and inorgani phosphat will at the pre ailing int a ll l r pH (6 4 7 0) release H and f rthe d cr ase pH_i (pK_a 6 1 f gluco 1-P gluc se 6-P fructo 6-P and pK 6 8 f H_2PO_4) Exces ive accumul tion f fructos 6-P would also pa tly ove come the pH inhibition f PFK (Triv di & Danforth 1966) with a furthe production f l cti acid as sult Thus it also would be f phy iol gical importance to have an inhibition f phosphorylase t low pH

Glycogen phosphorylase exi ts in two f rms *a* and *b* which are int convertibl To obtain maximum tivity t l v t d l vel f glucos 6-P t have maximum cap ity f accel tion f lact te f rmation the enzyme should be in the *a* f rm The conversion f phosphorylase *b* to phosphoryl e *a* i in muscle egul ted by very complex mechanism involving met boli as well as hormonal and nervous control It has been hown by Danf rth (1965) that the con ve ion f phosph ryl *b* t phosphoryl se *a* duri g timul tion f mus l i inhibited t low pH The pH inhibition f the con ve ion f pho phoryl se *b* t *a* could be due to inhibiti n f pho phorylase *b* kinase and ad yl y lase which are involved in the egul tion process and whi h activiti pH se sitive (Krebs et l 1964 Hawatari t l 1974) It must howeve be emembe ed that the ff t f pH on these enzyme was inve tig ted in vitr and the phy l gi l importan emains to be proved by in ivo xpe iments

The i hibition f PFK by pH can lso t some t nt be ove ome by an i se i AMP (T ivedi & Danfo th 1966) In this ituation (high AMP low pH) the acti ity of AMP deamina e which ha marked

optimum at pH 6 1–6 5 (Setlow & Lowenstein 1967) will increase and deamination of AMP to IMP will occur Deamination of AMP could the f re be of importance by preventing exc ssive AMP formation which would stimulate glycolysis nd re ult in a further acidification Deamination of AMP to IMP will also release ammonium i ns and consequently absorb part of the excess hydrogen ions

Hydrogen ions r involved in the lactate dehydrogenas reaction:



pyruvate + NADH + H^+ ⟷ lactate NAD

If equilibrium is assumed the following equati n can be derived:

$$\frac{(\text{la tat })}{(\text{pyruvate})} = K_{eq} \frac{(\text{NADH})(\text{H })}{(\text{NAD})} \qquad \text{Eq 6}$$

The increased la tate/pyruvate ratio observed aft r exercis (II) is due to inc eased NADH/NAD rati and to increased H^+ concent tion This pH dependence will occur in all a tions involving NADH/NAD and the bserved increase of the r tio α-glycerol phosphate/dihydroxyacetone pho phate ft r exe ci (Bergström et al 1971) should be viewed in this context

The importanc f pH for the creatine kina reaction and cont ts of high-ene gy phosphates has l ady been ext iv ly dis ussed (Chapters 4 & 5)

The ffect of pH on the mitochondrial function has been inve ti g ted by Mitchelson & Hi d (1973) and Tobin et l (1972) They showed that xidative phosphoryl tion was r the unaffected by the extramit chondrial pH in the range f 6 5–7 0 where seve inhi bition was btained t pH 6 0 (Mitchelson & Hi d 1973) The absen of physi logi al ca bon di xide tensions in these exp iment makes howeve an int rpr t ti n to an i vivo situation difficult Change in PCO_2 and HCO_3^- might have en eff t on the citric acid ycle (Adle 1970) and p esence of the CO_2 sy tem could lso be prerequi ite f btaining a pH change within the intramitochond i when the pH f the surrounding medium is changed Thus further experiments re n ce sary to xplor the fun ti of mitochondri at the low pH prev iling fte exhaustive exercise

Muscl contr tion is believed t b i itiated by a l ase f Ca^2 from the ar oplasmic r tic lum i to the cytoplasm which stimul t my sin ATP and causes the b akd wn of ATP and thus the f rmation f ross-linkage It has been shown that the

maximum ATPase ctivity f the actomyo in sy tem dec ease by bo t 25 % when pH decreas s from 7 0 to 6 5 (Schädle 1967 Portzehl et al 1969) The ame thors did also show that a dec ea in pH increased the amount of equired Ca^{2+} f r btaining maximum ti v tion In a tudy by Nakamaru & S hw tz (1972) the prot i binding of Ca^{2+} in the sar plasmatic ticulum w found t increase when pH i the incubation medium d c ed to 6 5 The in vitro studies sugge t that pH_i might be f importance f r th mus le contracti n i itself A dire t inhibition of the ontraction pro ss by H d ring severe l cti a ido i wo ld b an advantageous feed-ba k ignal f r the kel tal mus le by which the energy production would d c eas and f the cidification pr ented

The amount f nergy whi h i libe ted when 1 mol f ATP is hydrolyzed t ADP and P_i i d pend nt upon th con ent ation f ATP ADP P_i free Mg^2 and H Th hanges in the a f t rs whi h ccur d ing ha d ex rcise all tend to d c a the e rgy (IV) From th exi ting conditions in mu l it was calcul t d (IV) that the fre e rgy chang which oc urs wh n 1 mol ATP i hyd lyzed d creases from 54 kJ at t t 50 kJ aft exhaustiv xe ci The ATP/ADP ti used in these cal l tions was bt i d 4-6 sec afte t rminati n of x i (th time bef re the mus le ampl s were f zen) The v lue d ing the actual cont tion might be on id ably lower and the i fluence on the f ee en rgy hang thus even mo p onounced

The minimum nergy whi h i req ired to break and construct new bond between my i and a tin i not known It is howeve po ibl that the en gy whi h i btained from hydroly is f mol cul f ATP in some situations dec ease below thi l vel In thi ituation muscl ont ti tops not becaus of l ck f ATP but bec f too low ene gy yi ld i the ATP⟶ADP tran f ation It i well known that the whol ATP content pr sent in the mus l ll annot be tilized du ing contr tion Thi has been i t rpret d as a consequenc f omp rtment lization of ATP (Gercken & Schl tt 1968 Gudbj rnsson t l 1970) but an according to the bove di cussion s well be the consequenc f a too low energy yi ld in the ATP hydroly tion lt rnatively the consequenc of pH i hibition f the ont cti process

Chapte 8

SUMMARY

1 Methods have been developed f meas rements f intramuscular pH Total muscle pH was measured with a gl s lectrode in homogeni ed tissue Intracellul r pH (pH_i) was derived from meas rements f acid labile CO_2 in muscl samples

2 Normal values for resti g quadric ps muscle of man were: t tal muscle pH 7 080 ± 0 034 (n=12); pH_i 7 00 ± 0 06 (n=13)

3 Aft r fatiguing isomet ic cont cti n t 68 % of the maximum voluntary contraction fo c tot l muscl pH d creased to 6 56 - 0 07 (n=8)

4 Aft r exha stive bicy le ex rcise for about 5-10 min t tal muscl pH d c eased to 6 60 ± 0 14 (n 9) and pH_i to about 6 4 After 20 min covery total muscle pH as well as pH_i we re t red t the values t est The obtai ed changes in intramuscul r pH aft exercise and during reco ery as measured with the two diffe ent t chniques were in ag ement

5 Decrease in t tal muscle pH was f und to b linearly r lated to a cumulati n of l t te pyruv t (mmol/kg dry wt)
Isomet i ex rcise
pH = 7 06 0 00532(lact t + pyruv te -0 96 n=24
Bicycl exe ci (5-10 min):
pH = 7 06 - 0 00413(l ctat pyruv te); r=0 92 n=13
The differen between isometri and dynamic exer is was c nsidered mainly to be due t an fflux f H to the blood (s entially as carboni cid) during bicycl exe cise

6 Measurements in rte i l blood showed an identical incr e in lactate + pyruvat and ba deficit during exe cise indicating that efflux of lactate from muscl was ccompani d by an equimolar amount f H During the fir t minutes f recovery an increase in base deficit occurred in rt rial

blood whe ea no corr sponding change was found in lactate pyruvate content The esults are believed to be the consequenc f relea e f H in e ces of 1 ctat f om muscle during the first minut f recovery Measurements in muscle we e in accordance with thi hypothesis

7 The contribution f diff rent substances to the intr llular buff ing toward 1 ctic id d ing bicycl exercise has been e timated PCr depletion constitute 38 % efflux of carboni cid 18 % P_i 11 % and formation f glutamine 11 % of th buff ring The calculated total intr cellular buffer capa ity (68 91) is in agreeme t with in vitro measurements report d in lit atur and is leo v ry 1 t the tio −Δ(lactat pyruvat /Δ pH as btained afte bicycl exercise

8 Int acellula HCO_3^- wa calcul ted to 10 2 − 1 2 mmol/1 H_2O at re t and to bout 3 mmol/1 H_2O aft r exhaustive bicycl exerci

9 The r tio of ATP t ADP in mu le sample taken immedi tely aft t rmination f exe cise appe red t decre linearly when lact t content inc eased The d reas in ATP/ADP r tio wa believed to be the consequence f a teady-state condition d termined by decreased pH_i /and 1 ck f xygen

10 A gen l r l tion between content f pho pho reatin (PCr) and l tate was ob rved in muscl samples taken immediat ly aft termination of exer ise The l tion seemed to be independent of type intensity and dur tion f the preceding exe cise and i believed t be the consequence f a te dy-stat condition d t rmined by pH_i and lack f oxygen

11 No resynthe is f PC occurred when the blood supply to the muscl was clud d

12 T tal ad nine nucleotid (TAN) content in mus l decreased by about 15 % fte xhaustive bicycl exercis A corresponding increase in ino ine 5-monophosphat (IMP) was bserved Aft 30 min recovery the basal content f TAN was re to ed and IMP had disappeared from musel

13 The mass action rati of the creatine kinas reaction (MAR_{CK} (Cr)(ATP)/PCr)(ADP)) increased in muscle from about 5 at rest to about 100 after exhaustive exercise A f st recovery ccurred when the blood supply was intact The changes in MAR_{CK} in association with exercise were considered to be dependant upon pH_i and availability of xygen The possibility of eq ilibrium or a steady-state in the creatine kinase reaction is discussed

14 The regulation f energy met bolism at low pH_i is di cussed

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 456

EFFECTS OF GASTROINTESTINAL HORMONES ON CONCENTRATING FUNCTION AND MOTILITY IN THE GALLBLADDER

An experimental study in the cat

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ACTA PHYSIOLOGICA SCANDINAVICA
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This thesis is based on the following publications

I. An experimental method for studying *in vivo* gallbladder absorption
Svanvik, J and R. Jansson.
Gastroenterology 1977 72 634–638

II. Effects of intravenous secretin and cholecystokinin on gallbladder net water absorption and motility in the cat.
Jansson, R. and J Svanvik.
Gastroenterology 1977 72 639–643

III. Effects of intravenous vasoactive intestinal peptide (VIP) on gallbladder function in the cat.
Jansson R., G Steen and J Svanvik.
Gastroenterology 1978 In press.

IV A comparison of glucagon gastric inhibitory peptide and secretin on gallbladder function, formation of bile and pancreatic secretion in the cat.
Jansson R. G Steen and J Svanvik.
Submitted in *Scand. J Gastroenterol.*

V Effects of intraduodenal acid on gallbladder net water absorption and motility in the cat.
Jansson R.
Scand. J Gastroenterol. 1978 13. 209–215

These papers will be referred to in the text by their Roman numerals.

CONTENTS

INTRODUCTION

The gallbladder has two important physiological functions. Firstly the mucosa of the gallbladder absorbs water and electrolytes from the bile rendering the gallbladder contents a concentrated solution of organic constitutents. Secondly it stores the bile until the smooth muscle of the gallbladder contracts in response to a meal delivering concentrated bile to the intestine. These two main functions are *per se* extensively well studied. Present knowledge of the motility of the gallbladder has been reviewed by Lin (1975) and the concentrating function by Dietschy (1966) and Wheeler (1971).

Due to the accessibility of the organ the gallbladder has been frequently used for *in vitro* studies of transport mechanisms through biological membranes. However in a situation in which an organ is isolated from its blood circulation and lymphatic drainage and from the influence of nerves and blood-borne factors, its function cannot be fully comparable to *in vivo* conditions. Studies on the concentrating mechanism of the gallbladder *in vivo* are few and little attention has been focused on factors influencing the rate of water absorption in the gallbladder and thereby the concentrating effect on its contents. It is generally believed that the only important factor determining the extent of concentration of the bile is the time it remains in contact with the gallbladder mucosa. Experimental studies 45 years ago however have demonstrated that there seems to be a diurnal variation in the rate of gallbladder water absorption, suggesting a physiological regulation of its concentrating mechanism (Johnston et al. 1932). It has later been suggested that this regulation is mediated via hormones and autonomic nerves (Diamond 1968) but this has so far not been experimentally proved.

During the last decade our knowledge about the gastrointestinal peptides or hormones has increased tremendously. Besides the three classical hormones, gastrin, secretin and cholecystokinin, several peptides, called "candidate hormones" have been found. A lot of these peptides are known to influence water and electrolyte secretion and motility in the gastrointestinal tract.

Anatomy

The gallbladder in the cat is located below the surface of the right part of the clefted liver often with a broad connection to the liver. In rare cases it has an intrahepatic position. The cystic duct is long and winding, usually ending in the common bile duct but anomalies are frequent. The common bile and the pancreatic ducts share a common channel before emptying into the duodenum.

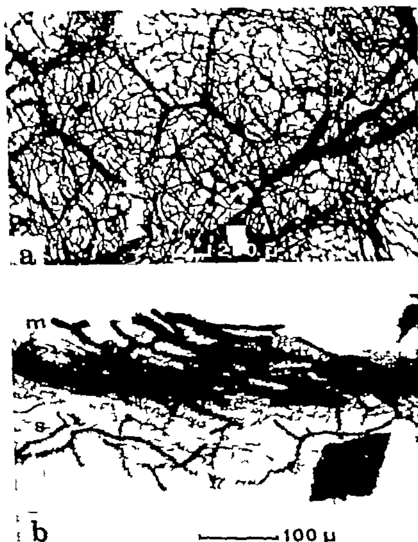


Fig. 2. Section from cat gallbladder showing the vascular anatomy after *in vivo* infusion of carbon black into the coeliac artery. The tissue was treated according to Spalteholz (1888). (*a*) The gallbladder wall is seen from the inside through all layers and their vasculature superimposed upon each other. Note the rich vasculature and the polygonal arrangement of the vessels. (*b*) A cross section (100μ) of the gallbladder wall. The mucosal layer on top. The difference in density of the vascular network between mucosal (*m*) and subserous (*s*) layers is obvious.

The sections were prepared in collaboration with Dr Mikael Romanus, Laboratory of Experimental Biology, Department of Anatomy, University of Goteborg.

and Boyden 1943 Kune 1972) The vagal fibres are predominantly found in the hepatic branch of the right vagal trunk and the sympathetic fibres derive from the celiac plexus. In the gallbladder wall two plexuses of nerves can be identified one in the subserosa and one plexus in near contact with the epithelial cells (Newton 1964 Sutherland 1967)

Physiology

The bile that enters the gallbladder from the liver is a solution comprising of about 97 per cent water and 1 – 2 per cent bile acids. The remainder is mostly phospholipids, cholesterol bile pigments and water-soluble salts (Scratcherd 1965 Wheeler 1968 Brooks 1976). The electrolyte composition of the bile differs between species and also within a given species dependent on the time of collection Cations concentrations in bile are roughly similar to that in plasma. There are greater differences in anions concentrations, and the dominant anion chloride is somewhat lower than that in plasma. The concentration of bicarbonate is often higher giving the hepatic bile a higher pH than that in plasma. Hepatic bile is isoosmolar to plasma

In the gallbladder as much as 90 per cent of the water in bile can be removed (Rous and McMaster 1921 Ravdin et al. 1932) and the gallbladder epithelium maintains per tissue weight one of the highest rates of water transport (Diamond 1968). The concentrating process involves a reabsorption of inorganic electrolytes chiefly sodium chloride and sodium bicarbonate The result is a progressive increase in the concentration of the conjugated bile acids and reduced concentrations of chloride and bicarbonate The final out come the concentrated gallbladder bile is a solution with high concentrations of bile acids. Sodium, potassium and calcium concentrations are high compared to plasma and chloride and bicarbonate concentrations low With the pronounced increase in all solutes in the bile an increase in the osmotic activity could be expected. However conjugated bile salt anions form osmotically inactive micelles, explaining why the bile remains isoosmotic with serum (Johnston and McBain 1942)

Gallbladder absorption. The transport mechanism in the gallbladder wall has been extensively studied *in vitro* (Diamond 1962 Wheeler 1963 Dietschy 1964). The most reasonable theory for the mechanism of absorption is the Curran serial membrane model (Curran and McIntosh 1962). This includes an active transport of solute across the lateral cell walls of the epithelial cells into the lateral spaces. A local osmotic gradient is created which forces water to pass from the lateral cell wall into the lateral space The hydrostatic pressure in the lateral space rises and drives the solution out through the basal clefts. This theory is supported by electron microscopy studies showing distended intercellular spaces at high absorptive states (Kaye et al 1966).

The rate of fluid absorption from the gallbladder lumen *in vivo* has been studied by different methods (for review se I) Ravdin et al. (1932) found that 3 – 4 ml/h or 16 per cent of the gallbladder olume per hour was absorbed by the canine gallbladder

Grim and Smith (1957) estimated canine water flux rates to about 10 per cent of the gallbladder volume per hour. The rabbit gallbladder has been shown to absorb fluid at rates of 0.8 – 1.0 ml/h or about 30 per cent of gallbladder volume per hour (Whitlock and Wheeler 1964). The rate of fluid absorption reported in studies on the isolated organ is slower. Whitlock and Wheeler (1964) observed that the absorptive rate in rabbit gall bladders was about one third of the rate observed *in vivo*. However, Ostrud et al. (1967) studied the rate of fluid transfer across human gallbladder removed for cholelithiasis and reported rates of absorption as high as 2.9 ml/h or approximately 10 per cent of gallbladder volume per hour.

Substances other than water and electrolytes are only to a small extent absorbed. Ostrow (1967) found that unconjugated bilirubin was absorbed in the gallbladder of the guinea pig. The mechanism was thought to be passive and dependent upon water absorption. In healthy gallbladders bile acids are not absorbed, whereas during inflammation rapid absorption can be seen (Ostrow 1969, 1971). Phospholipids and cholesterol are also absorbed to a small extent (Riegel et al. 1932, Neiderhiser et al. 1976).

Johnston et al. (1932) reported that the average hourly absorption of water in the canine gallbladder was three times greater in daytime than at night and Potter (1936) made the observation that the gallbladder in pregnant women undergoing cesarean section was distended, containing bile with much the same concentrations as hepatic bile. The altered function of the gallbladder during pregnancy has been found also in animals (Smith et al. 1941, Myers and Hill 1942). These findings have been explained by the influence of hormones and autonomic nerves (Diamond 1968).

The effects of humoral factors on the concentrating function of the gallbladder have been studied, however, mostly *in vitro*. The results using different experimental models and in different species are often conflicting. Thus, Cremaschi and Galante (1969) reported that vasopressin *in vivo* slightly increased but *in vitro* inhibited net water transport. Furthermore, oxytocin produced a complete inhibition of water transport *in vitro* (Diamond 1962) whereas no effect could be revealed *in vivo* (Cremaschi and Galante 1969). Peskin et al. (1969) studied the effects of gastrin, cholecystokinin (CCK) and secretin on isolated canine gallbladders and found a reduced fluid transport when one of these hormones was added to the mucosal bath. Kraft et al. (1969) could not find any effect of CCK and gastrin on isolated rabbit gallbladders, whereas secretin reduced the fluid transport. The effects of gastrin, CCK and secretin on the concentrating function of the gallbladder *in vivo* so far have not been studied. The effects of other gastrointestinal peptides, vasoactive intestinal peptide (VIP) and gastric inhibitory peptide (GIP) have not been reported, neither *in vivo* or *in vitro*.

Little is known about nervous influence on the concentrating function of the gallbladder. Diamond (1962) showed that acetylcholine caused a small inhibition in the net water transport by the fish gallbladder. Westphal et al. (1931) found that electric stimulation of the distal end of the right vagus nerve in the anesthetized dog caused an increase in the reabsorptive function of the gallbladder.

Gallbladder motility. According to Ivy (1934) the gallbladder has two types of motor activity: tonus rhythm and tonic contraction. The tonus rhythm is maintained by a rhythmical contraction and relaxation occurring, in the cat at a rate of about one contraction a minute. The tonic contraction seen after a meal gives a sustained rise in intragallbladder pressure persisting from five to thirty minutes or more. It is generally believed that CCK is mainly responsible for this contraction and the effect of this hormone on gallbladder motility is thoroughly studied (Jorpes and Mutt 1973).

The mode of action of CCK on the gallbladder is not known. The effect is neither modified by vagotomy (Scheinin et al. 1967) nor by atropine, α- and β-adrenergic blockers (Tooull and Watts 1972, Lin 1975). It has been concluded that CCK acts directly on gall bladder muscle. An earlier suggestion (Amer 1974) that the action is mediated by increasing the concentration of cyclic GMP has recently been disproved (Andersson et al. 1977). Among hormones, besides cholecystokinin, secretin and glucagon have no effects alone on gallbladder contraction while they augment contractions induced by CCK (Vagne and Troitskaja 1976). VIP has been found to decrease CCK-induced contractions in the feline gallbladder (Vagne and Troitskaja 1976) and relaxes the gallbladder of the opossum (Ryan and Cohen 1977).

Results so far published describing the effects of autonomic nerves on gallbladder motility are contradictory. Bainbridge and Dale (1905) found a relaxation of the gallbladder in response to electrical stimulation of the splanchnic nerve. Pallin and Skoglund (1964) found no effect and Sadler et al. (1972) reported a distinct contraction of the gall bladder. Studies on human and cat gallbladder muscle have shown that sympathetic α receptor stimulating agents have a weak contractile activity, whereas β-receptor stimulants relax the muscle strips (Persson 197_). Electrical stimulation of the parasympathetic nerves causes gallbladder contractions both in dogs and cats (Pallin and Skoglund 1961, Satler et al. 197?). Acetylcholine causes a marked contraction *in vitro* which is antagonized by atropine (Jung and Greengard 1933) whereas atropine itself causes varying degree of relaxation (Westphal et al. 19_3, Satler et al. 197?).

AIMS OF THE PRESENT INVESTIGATION

The absorptive function of the gallbladder as well as gallbladder motility *per se* have been thoroughly investigated. However most studies are based on *in vitro* experiments. Few investigations describe factors regulating the rate of fluid transport in the gallbladder. Little is known about the actions of secretin and newer gastrointestinal peptides on gallbladder motility.

The aims of the present investigation were

1. to work out a method for *in vivo* measurement of net transport of water and electrolytes across the gallbladder wall and concomitant motility in the gallbladder

2. to study the *in vivo* effects of secretin, cholecystokinin, vasoactive intestinal peptide, gastric inhibitory peptide and glucagon on gallbladder concentrating function and motility

3. to evaluate the effects of intraduodenal acid, a factor known to release gastrointestinal hormones, on gallbladder net water absorption and motility

Gallbladder motility According to Ivy (1934) the gallbladder has two types of motor activity tonus rhythm and tonic contraction The tonus rhythm is maintained by a rhythmical contraction and relaxation occurring, in the cat at a rate of about one contraction a minute The tonic contraction seen after a meal gives a sustained rise in intragallbladder pressure persisting from five to thirty minutes or more It is generally believed that CCK is mainly responsible for this contraction and the effect of this hormone on gallbladder motility is thoroughly studied (Jorpes and Mutt 1973).

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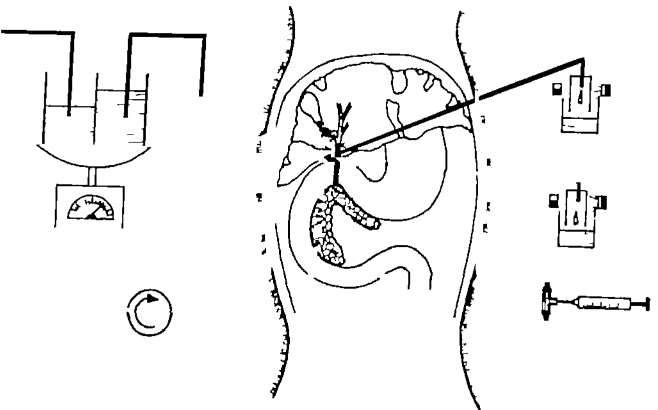


Fig 3 Schematic illustration of the technique used for studying net water absorption from the gallbladder *in situ*. The gallbladder is perfused at a constant rate from neck to fundus. Net water absorption is registered as a continuous decrease of the weight on an electromagnetic balance. The syringe is connected to the inflow catheter to ensure mixing within the gallbladder. Hepatic bile flow and pancreatic secretion are registered with drop recorders.

mixing within the gallbladder 0.5 ml of the perfusate was aspirated and reinjected at 5 min intervals with a syringe connected to the inflow catheter Every 20 min the total amount of effluent fluid was aspirated from its chamber on the balance and the same amount of fresh perfusate added to the influent chamber In this way no fluid recirculated The concentration of the tracer in the perfusate was measured in a liquid scintillation spectrometer Net water transport was calculated according to the formula

$$\text{Absorption} = Q_{in} - \frac{Q_{in} \quad C_{in}}{C_{out}}$$

Q_{in} = volume rate of inflow

C_{in} = tracer concentration in the inflow

C_{out} = tracer concentration in the outflow

The formula was considered to be valid during "steady state" conditions one hour after any induced change when the perfused volume was some three times the volume in the gallbladder lumen.

Measurements of electrolyte transport

The concentrations of sodium, potassium, chloride and bicarbonate in the influent and effluent perfusion fluids were measured (III IV). Knowing these concentrations, the perfusion rate and the net water absorption the net transport of electrolytes across the gallbladder wall could be estimated according to the following formula:

$$\text{Absorption} = Q_{in} \quad C_{x_{in}} - Q_{out} \quad C_{x_{out}}$$

Q_{in} = volume rate of inflow

Q_{out} = volume rate of inflow minus net water absorption calculated with the PEG-method

$C_{x_{in}}$ = concentration of the solute in the inflow

$C_{x_{out}}$ = concentration of the solute in the outflow

The formula was considered to be valid at "steady state" conditions one hour after any induced change

Measurements of gallbladder motility

Changes in the weight of the balance reflect changes in volume in the perfusion system either due to an absorption or a secretion in the gallbladder or displacements of fluid due to a contraction or a relaxation in the gallbladder (I). Since the net water absorption is estimated by the PEG-method changes in gallbladder volume can be calculated.

In paper I and II gallbladder motility was also recorded in separate experiments by a direct method. A balloon of thin rubber attached to the end of a plastic cannula was introduced into the lumen of the gallbladder The balloon was filled with saline and connected to the electromagnetic balance The hydrostatic pressure was set at 7 cm H_2O Weight changes on the balance reflected variations in gallbladder volume

Recordings of hepatic bile flow and pancreatic secretion

The common bile duct and the pancreatic duct were cannulated at their insertion into the duodenal wall and flow was continuously measured by optical drop recorders.

Hormones

Pure Secretin and 20 % pure CCK were purchased from the Gastrointestinal Hormone Laboratory Karolinska Institutet, Stockholm, Sweden
VIP was a gift from Professor V Mutt Stockholm, Sweden.
GIP was a gift from Dr J C. Brown Vancover Canada.
Glucagon was purchased from Novo Industri A/S Copenhagen Denmark. All hormones were stored in crystalline form. They were kept frozen until just before the experimental period when they were dissolved in saline and infused into a femoral vein.

Perfusion solutions

Six different solutions were used to perfuse the gallbladder

1. Hepatic bile collected from other cat experiments (I – III V).
2. Gallbladder bile aspirated from the gallbladder in animals deprived of food for at least 24 hours (I).
3. Saline (0.9 g/l) (I).
4. Saline isotonic to plasma (I – III V).
5. Electrolyte solution with concentrations of sodium, potassium, chloride and bicarbonate the same as those in hepatic bile and buffered to isoosmolarity by mannitol (IV Na^+ 130 K^+ 5 Cl^- 105 HCO_3^- 30 mmol/l)
6. Mannitol solution (I)

METHODOLOGICAL CONSIDERATIONS

The method to evaluate the net water transport in the gallbladder involves a number of both practical and theoretical problems. The technique assumes that there is no leakage from or to the perfusion system. The existence of accessory bile duct connections to the gallbladder was not found nor any leakage from the system outside the gallbladder (I). The method also assumes a complete mixing in the gallbladder which *per se* is difficult to assess. The volume method also assumes a constant density of the perfusate during its passage through the gallbladder. This assumption has also been tested.

During conditions with a constant gallbladder volume the prerequisites for the technique seem to be fulfilled. The two different methods for measuring net water transport are then highly compatible (I).

With a constant gallbladder volume the formula used for the calculation of the net water transport with the help of PEG is derived as follows.

$$\begin{cases} Q_{in} \;\; C_{in} = Q_{out} \;\; C_{out} \\ Abs = Q_{in} - Q_{out} \end{cases}$$

$$Abs = Q_{in} - \frac{Q_{in} \;\; C_{in}}{C_{out}}$$

Q_{in} = volume rate of inflow

Q_{out} = volume rate of outflow

C_{in} = tracer concentration in the inflow

C_{out} = tracer concentration in the outflow

This formula seems also to be valid if there is a continuously changing volume in the gall bladder providing that the mixing procedures abolish gradients in the tracer concentration between the lumen and the outflow as well as within the lumen.

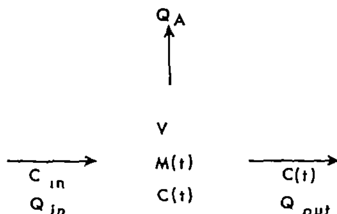
$$\begin{cases} Q_{in} \;\; C_{in} = C_{out} \quad (Q_{out} + Q_{\Delta V}) \\ Abs = Q_{in} - (Q_{out} + Q_{\Delta V}) \end{cases}$$

where $Q_{\Delta V}$ = the flow to change the gallbladder volume

$$Abs = Q_{in} - \frac{Q_{in} \;\; C_{in}}{C_{out}}$$

The formula used to calculate the net water absorption is thus valid even if the volume of the gallbladder is not constant.

The major disadvantage of the present technique using PEG is that changes in net water transport in the gallbladder are detected after considerable delay due to the slow perfusion rate. To account for this factor an equilibration period of at least one hour had to be used before a new absorptive state was considered to be representative. The size of this error can be calculated.



Q_{in} = volume inflow

C_{in} = PEG concentration in the inflow

V = volume of the gallbladder

M (t) = amount of tracer in the gallbladder

C (t) = PEG concentration in the gallbladder

Q_A = net water absorption

Q_{out} = volume outflow

In such a system where a volume V is perfused and in which complete mixing is ensured the change of tracer ΔM (t) during time Δt can be expressed as

$$\Delta M(t) = Q_{in} \quad \Delta t \quad C_{in} - Q_{out} \quad \Delta t \quad C(t) \quad \text{where}$$

$$C(t) = \frac{M(t)}{V}$$

These equations can be solved

$$C(t) = C_{in} \frac{Q_{in}}{Q_{out}} \quad (1 - e^{-t \quad Q_{out}/V})$$

If there is a stepwise change in the net water absorption in the gallbladder the concentration of PEG in the outflow will change exponentially according to this formula. If V = 3 ml and the net water absorption stepwise changes it can be calculated that the change in PEG-concentration in the outflow after 70 min is between 0.86 and 0.93 of the final value dependent of flow. Using these factors it can be calculated that the estimation after 70 min of the net water absorption is 0.88 ml/h when it stepwise rises from zero to 1 ml/h. When a secretion of 1 ml/h is induced from a state of no net transport across the wall this is after 70 min estimated to be 0.92 ml/h. This error should be considered, especially when comparing the results from the PEG-method with the results from the volume registering method. However often more than 60 min after an induced change passed before the start of the period used for the estimation of net water transport and the error then is smaller.

Since the gallbladder was perfused for several hours the physiological condition of the preparation can be discussed. In order to evaluate this factor the microscopic morphology of eight gallbladders taken from three different types of experiments were studied. The gallbladders were removed after the experimental runs, fixed and processed for examination by light microscopy. Each gallbladder was studied in several sections stained with hematoxylin-eosin. The three different types of experiment were firstly perfusion with isotonic saline solution (? experiments), secondly perfusion by hepatic bile (3 experiments) thirdly control gallbladders taken from other cat experiments after surgical preparation of the stomach but with no intervention into the biliary tract (3 experiments). It was found that perfusion of the gallbladder with isotonic saline during eight hours caused a moderate acute inflammatory reaction throughout the gallbladder wall (fig. 4 left). When the gallbladder was perfused by hepatic bile no reaction was noted in the inlayers of the gallbladder (fig. 4 right). However in the subserosal coat there was an infiltration of polymorphonuclear leucocytes. At higher magnification pavementing of leucocytes was seen in the vessels. The same picture was found in the control gallbladders. Therefore it is not likely that this reaction is due to the perfusion *per se* but is secondary to the operative trauma.



Fig. 4 Sections of the wall from cat gallbladders perfused with different solutions. Magnification x 300 (*Left*) From a gallbladder perfused during eight hours with saline solution Note signs of acute inflammatory reaction with a great number of polymorph-nuclear cells (*Right*) From a gallbladder perfused during eight hours with hepatic bile The histologic picture is almost normal

RESULTS AND DISCUSSION

Basal gallbladder function

Water absorption. The techniques described were used to study the basal net water absorption from different fluids perfusing the gallbladder lumen. These results are shown in table I The gallbladder volume at the present conditions was estimated to average 2.3 ml (I). The rate of reabsorption of water from hepatic bile in the gallbladder lumen was 0.7 ml/h corresponding to 30 per cent of the gallbladder volume. The net water absorption from concentrated bile was only 0.23 ml per hour demonstrating a decreasing rate of net water absorption as the gallbladder concentrates its contents.

Table I A comparison of net water absorption from different perfusion fluids in the gallbladder lumen. (The solutions are described on page 15).

	Water absorption (ml/h ± SE)		
	Volume registering method	PEG method	Number of experiments
Hepatic bile	0 68 ± 0.06	0.67 ± 0.06	21
Gallbladder bile	0 23 ± 0 11	–	4
Saline	1.08 ± 0 17	–	7
Isotonic "saline"	1.08 ± 0 10	1 06 ± 0 11	19
Electrolyte solution	0.87 ± 0 13	0 99 ± 0 15	13
Mannitol solution	0 03 ± 0.03	–	5

These results can be compared to earlier reports in the literature Ravdin et al. (1932) found in their classical work the rate of fluid absorption in the canine gallbladder *in vivo* to be approximately 16 per cent of the total gallbladder volume per hour Whitlock and Wheeler (1964) studied the rabbit gallbladder *in situ* and found absorption rates of about 30 per cent of gallbladder volume per hour Gerolami and Sarles (1968) studied water

absorption from the feline gallbladder *in vivo* using an experimental model where bile was continuously infused into the gallbladder The residual contents after a period of 15 to 4 hours were determined and an average net water absorption of 0.34 ml per hour can be calculated from their results. The low value in their study can be explained by the fact that there is a gradually increasing concentration of bile acids in the gallbladder which in itself diminishes water absorption (Ammon 1974)

The net water absorption from the gallbladder during perfusion with electrolyte sol utions can also be seen in Table I The mean values are higher compared to the rate of fluid absorption from hepatic bile This observation is in accordance with that of Ravdin et al (193) who found that the rate of absorption in the canine gallbladder was 8 ml/h from a bile free solution and 6 75 ml/h when the gallbladder contents consisted of he patic bile This higher absorption rate can be explained by the absence of bile acids that are known to damp the net water absorption (Ammon 1974). Another explanation is that the sodium ions in these solutions are not bound in micelles and a higher concentra tion of free sodium ions is exposed to the mucosal cells.

Addition of bicarbonate to the bile free perfusion solution (the electrolyte solution containing HCO_3 30 mmol/l see page 15) did not change the rate of fluid transport. This observation contrasts with earlier *in vitro* findings. Diamond (1964) and Wheeler et al (1969) reported twice as rapid fluid transport by the gallbladder in bicarbonate-con taining media compared to bicarbonate-free solutions. A possible explanation for the dif ference *in vitro* compared to *in vivo* is that the bicarbonate ion affects the utilization of endogenous substrate necessary for fluid transport (Martin 1974). *In vitro* the intracel lular concentration of bicarbonate is dependent on the concentration of this ion in the bathing solution whereas *in vivo* the intracellular concentration is dependent on the blood concentration.

Removal of all electrolytes from the perfusion solution gave a complete loss of net water absorption This is well-known earlier shown *in vitro* by Dietschy (1964), and de pending on the fact that water transport is secondary to an active anion-cation transport (Diamond 1962)

Electrolyte composition in hepatic and gallbladder bile. Concentrations of sodium, po tassium chloride and bicarbonate were determined in both hepatic bile and gallbladder bile Hepatic bile was collected from the common bile duct in anesthetized animals de prived of food for 24 hours. Gallbladder bile was obtained by puncturing the gallbladder of cats also deprived of food for at least 24 hours. These results are summarized in Table II.

The results are in accordance with those found in man and dog (Brooks 1976 Diamond 1968) It can be concluded that the ultimate product of the concentrating process in the gallbladder is a solution in which concentrations of sodium and potassium are high compared to plasma and chloride and bicarbonate concentrations are low The pH de creases during the concentrating process.

Table II The electrolyte composition of cat bile expressed as mmol/l

	Na	K^+	Cl	HCO_3	pH
Hepatic bile (n = 7)	161 ± 3	3.9 ± 0.1	84 ± 2	40 ± 4	8.2 ± 0.1
Gallbladder bile (n = 9)	231 ± 9	7.8 ± 0.3	4 ± 1	4 ± 1 (n = 7)	6.5 ± 0.1 (n = 7)

Electrolyte absorption. The net transport of electrolytes across the gallbladder epithelium could be estimated by measuring the concentrations of the ions in the influent and effluent perfusion fluids and by knowing the perfusion rate and the net water movement. The average results from a group of experiments where the gallbladder was perfused by hepatic bile and another group perfused by an electrolyte solution (see page 15) can be seen in Table III.

Table III The average basal electrolyte absorption across the gallbladder wall

Perfused solution	Na µmol/h	K µmol/h	Cl µmol/h	HCO_3 µmol/h
Hepatic bile (n = 9)	98 ± 9	1.3 ± 0.3	102 ± 10	55 ± 12 (n = 7)
Electrolyte solution (n = 13)	162 ± 24	6.3 ± 0.7	174 ± 19	64 ± 11

It is evident that during "resting" conditions, there is a net absorption in the gallbladder of all ions measured, from both bile and from the bile-free solution. The higher electrolyte net absorption measured from the bile free solution can be explained by the absence of bile acids damping the absorption (Ammon 1974). Another explanation is that the ions in the bile free solution are not bound in micelles and higher concentrations of free ions are exposed to the mucosal cells

In agreement with the results of Ravdin et al (1932) an imbalance between the net absorption of measured anions and cations was seen in the present studies. Since electroneutrality is supposed to be maintained there must be another explanation of this imbalance On perfusion with bile changes in hydrogen ion activity and protonation or deprotonation of substances like bile acids proteins and phospholipids are not included in the balance Furthermore the absorption of some other cations or the secretion of some other anions, which were not measured could be involved However in the studies where

the gallbladder was perfused with a bile free solution an imbalance of the same magnitude was observed. In order to evaluate if this was due to a secretion of proteins, organic acids and phosphate concentrations of these substances were measured in the effluent (IV). It was found that the total content of organic acids and phosphate in the perfusate were less than 0.3 mmol/l and the concentration of protein was less than 0.01 mmol/l Thus, a secretion of these anions is unlikely to explain the whole imbalance The methodological errors are not supposed to be large enough to explain the whole cation anion imbalance *The final explanation is probably a combination of several processes such as the* secretion of an anion not measured into the gallbladder lumen and an overestimation of bicarbonate

Volume and motility The gallbladder volume at the basal conditions with a distending pressure of 7 cm of water was estimated to average 2.3 ml (I). During basal condition it was found that the volume of the gallbladder remained at a constant level In some experiments small spontaneous contractions were registered (I) However these did not displace more than 0 1 ml and did not disturb the stable gross appearance of the curve The registration technique did not usually allow recordings of short and transient minor contractions in the gallbladder

Gastrointestinal hormones and gallbladder function

Secretin. The main physiological effects of secretin are regarded to be the production of a watery bicarbonate-rich fluid from the pancreas and the liver Little is known about its effects on the gallbladder function. In the present study the effects of i v secretin (2 U = 0.6 µg per kg/h) on gallbladder function were tested, when the organ was perfused by saline an electrolyte solution and hepatic bile in three different series of experiments When the gallbladder was perfused by hepatic bile or the electrolyte solution secretin completely abolished the net water absorption and in some experiments a slight secretion was seen (II IV) The corresponding response for secretin when the gallbladder was perfused with saline was a significant secretion (II). Electrolyte absorption was studied in a series of experiments where the gallbladder was perfused with an electrolyte solution. The net absorption of chloride and potassium ions were significantly reduced and the net absorption of sodium and bicarbonate ions abolished (IV). Secretin was found not to influence the resting volume or the motility of the gallbladder but in all experiments it increased bile outflow and pancreatic secretion.

This study is an evaluation of *in vivo* effects of secretin on gallbladder *water transport.* It seems obvious that secretin in the given dose strongly influences the net transport of water and electrolytes across the gallbladder wall whereas *motility is not affected* When the gallbladder retained bile the net absorption of water was abolished but no secretion was seen However when saline was used as the perfusate, *secretin caused a significant* secretion of fluid into the lumen. This difference may be explained by the possibility that saline damages the gallbladder epithelium and *causes an inflammatory response. This* hypothesis is corroborated by histological findings showing oedema and infiltration of leucocytes in the mucosa of gallbladders *perfused with saline*

Since the plasma level of secretin in the present study was not measured it is difficult to assess whether it is within the physiological range The dose (2 U per kg/h) however was found to induce a pancreatic and biliary response of the same order of magnitude as duodenal acidification in the same type of experiments (V). Furthermore Way and Grossman (1970) reported that half maximal response for pancreatic secretion in cats was induced by 3.8 ± 0.5 U/kg/h of secretin Recent studies have been published when plasma secretin levels after exogenous administration have been correlated to maximal pancreatic response both in man and pig (Häckl et al. 1977 Schaffalitzky de Muckadell et al 1977) These authors found that maximum pancreatic output was obtained with an exogenous administration of about 1.0 U/kg/h and that an infusion of secretin 0 3 U/kg/h gave the same secretin plasma level as duodenal acidification

During the last few years the physiological role of secretin has been under debate since several laboratories have been unable to observe a postprandial secretin release (for ref see Straus 1978). The only known situation with raised plasma levels of secretin is during acid infusion into the duodenum (Rayford et al 1976 Fahrenkrug et al 1978) which is not strictly physiological. Straus (1978) reported in a critical review a wide range of measured steady state secretin plasma levels ranging from 0 6 pg/ml to greater than 600 pg/ml. Thus at present the true secretin fasting level cannot be given It can not be excluded that the dose of secretin used in the present study gives plasma concentrations over the physiological range

Cholecystokinin. The main physiological effects of CCK are to contract the gallbladder and to induce a secretion rich in enzymes from the pancreas. In the present study an venous infusion of CCK (2 U = 0.66 μg/kg/h) did not change the rate of fluid absorption in the gallbladder (II). Further it had a weak choleretic effect and induced a relatively low discharge from the pancreas. Two different methods were used to estimate the contraction of the gallbladder during infusion of CCK a direct technique with a balloon inserted in the gallbladder and an indirect method by subtracting the PEG value from the volume-registering value (see Methods). The average values obtained with the balloon technique were an evacuation of 18 % of the basal volume The corresponding figure with the volume registering method was 46 % of the basal volume The maximal contraction was reached 20 – 25 minutes after the start of the continuous infusion (II).

No earlier investigations have evaluated the effects of CCK on gallbladder water transport *in vivo* *In vitro* a slight decrease of the net water absorption has been reported when CCK is administered to the solution bathing the serosa of the gallbladder (Peskin et al. 1968). *In vivo* in the present study CCK had no effect on the water transport and this discrepancy illustrates the difference between *in vitro* and *in vivo* responses of the gallbladder

The strong contractile effect of CCK on the gallbladder has been extensively studied in man and animals (Ivy 1934) Park et al (1970) found that the gallbladder of healthy individuals contracts to 23 % of its initial volume 15 minutes after an i v injection of 1 U/kg of CCK and a fat meal expelled 55 % of the initial gallbladder volume after 15 minutes.

A number of studies using radioimmunoassay to estimate CCK levels have been report ed (for ref see Straus 1978). However there are big discrepancies between these results and hormone levels at basal conditions and after a meal are not yet known Therefore it is impossible to evaluate which dose of exogenous CCK that corresponds to the physiological release Debas and Grossman (1973) evaluated the dose of pure CCK required to induce a half-maximal response in pancreatic protein secretion in dogs to be 0 96 μg/kg/h which should be compared to 0.66 μg/kg/h administered in the present study

The discrepancies between the two methods used in the present study to evaluate gall bladder contraction may be explained by difficulties in completely filling out the whole of the gallbladder lumen with the balloon

Vasoactive intestinal peptide. Vasoactive intestinal peptide (VIP) isolated 1970 by Said and Mutt is chemically related to secretin, glucagon and GIP (Said and Mutt 1970) VIP is found in the whole of the gastrointestinal tract and the total amount of this peptide found there far exceeds that of gastrin and secretin (Bloom and Polak 1976) It still be longs to the group of "candidate hormones as its mode of release and physiological ac tions are not known (Grossman 1974 Bloom 1977) At exogenous administration VIP has a wide range of biological actions: systemic vasodilatation inhibition of gastric sec retion, relaxation of gastric and gallbladder muscle stimulation of pancreatic and bile secretion and stimulation of small intestinal secretion (Said and Makhlouf 1974).

In the present study it was shown that an intravenous infusion of VIP (1 μg/kg/h) re versed the net water transport in the gallbladder from an absorption to secretion both when the gallbladder was perfused by hepatic bile and saline (III). During infusion of VIP the net transport of sodium, potassium and bicarbonate also changed direction and a secretion into the gallbladder lumen was found. The absorption of chloride ions was significantly reduced but no secretion found When the infusion of the peptide stopped the absorptive function in the gallbladder returned to the preinfusion level

VIP caused an initial slight relaxation of the gallbladder amounting to around 10 per cent of the basal volume No subsequent changes in the gallbladder volume were seen during the later part of the VIP infusion periods. Bile flow was doubled and a pancreatic secretion of around 5 ml/h was seen in response to VIP

Recently Sundler et al. (1977) demonstrated with an immunohistochemical technique VIP-containing nerve fibres in the gallbladder wall of cat and man These fibres are located in the smooth muscle layer and just beneath the epithelial cells whereas no immunoreactive endocrine-like cells could be demonstrated. This special arrangement is parti cularly interesting since it has been postulated that VIP is a neurotransmitter (Bryant et al. 1976) and since it is known that the concentration of VIP in portal blood increases during electric vagal stimulation (Schaffalitzky de Muckadell et al 1977). These findings raise the question as to what happens in the gallbladder during electric vagal stimulation In a preliminary study it has been found that efferent vagal stimulation in the neck afte the administration of atropine causes a relaxation of the gallbladder and a significant de crease in the net water absorption (Svanvik et al. 1978 unpublished observations). Ex

periments are in progress to further study a possible release of VIP in the gallbladder Such a mechanism might produce a high concentration of the peptide locally at the receptors to an extent that is not reflected in the plasma levels

Gastric inhibitory peptide Gastric inhibitory peptide (GIP) was isolated in 1969 from impure CCK preparations by Brown et al. (1969) and was purified and sequenced two years later (Brown and Dryburgh 1971) Its main actions on i.v infusion are to inhibit gastric acid secretion (Pederson and Brown 1972) and release insulin (Dupre et al. 1973). Furthermore GIP seems to influence gastrointestinal motility (Pederson 1971) and reduce the net absorption of water and electrolytes in the human jejunum (Helman and Barbezat 1977)

In the present study the effects of GIP on gallbladder net water and electrolyte transport as well as gallbladder motility pancreatic secretion and bile flow were investigated. (IV) It was found that GIP intravenously at a dose of 1 – 30 μg/kg/h did not influence the net transport of water Na K Cl or HCO_3 and it had no effect on gallbladder motility or the secretion of bile and pancreatic juice The potency of GIP was tested in two experiments where the gastric volume was registered in acutely vagotomized animals. In both experiments a relaxation of the stomach could be registered at an i.v dose of 30 μg/kg/h.

GIP belonging to the same hormone family as secretin and VIP seems to influence human jejunum water and electrolyte transport, but does not share the effects of these hormones on the gallbladder A dose of the peptide that relaxes the stomach was not found to influence the volume of the gallbladder

Glucagon. Pancreatic glucagon has its main actions on carbohydrate metabolism (Unger and Orci 1976). Like secretin and GIP it inhibits gastric secretion and motility (Lin and Spray 1968 Stunkard et al 1955). In high doses glucagon can induce a relaxation of the human gallbladder and increase bile secretion in man (Dyck and Janowitz 1971 Chernish et al. 1972) In the present study it was found that i v glucagon at a dose (1 – 20 μg/kg/h) that significantly raised the blood glucose level did not affect the net transport of water or Na K Cl and HCO_3 in the gallbladder (IV). No changes in gallbladder motility were seen and bile flow and pancreatic secretion were also unaffected whereas secretin in the same experiments had a potent effect

Glucagon and secretin are chemically closely related. Secretin contains 27 amino acid residues, glucagon contains 29 and of these 14 occupy identical positions. In the stomach both inhibit gastric secretion but in the small intestine the effects of the two hormones are divergent Glucagon is known to reduce the intestinal net water absorption in man (Hicks and Turnberg 1974) and to induce a secretion into the lumen of the small intestine in the dog (Barbezat and Grossman 1971) Secretin does not seem to influence the intestinal fluid transport (Barbezat and Grossman 1971) The opposite situation seems to be valid for the gallbladder where secretin completely abolished the net water absorption (II IV) while glucagon did not affect the rate of fluid transport. Glucagon has been demonstrated to have a choleretic effect in man (Dyck and Janowitz 1971) and

dog (Jones et al 1971) while in the rat no increase in hepatic bile flow was registered (Shaw and Heath 1973) In the reported studies the choleretic effect of glucagon at a low dose seems to be slight transient or absent and in the present study no effect was seen (IV).

Gallbladder response to intraduodenal acid

Introduction of hydrochloric acid into the duodenum initiates a complex response involving the activation of autonomic nerves (Pavlov 1902 Tanturi and Ivy 1938) and release of gastrointestinal hormones (Starling and Bayliss 1902, Wormsley 1971). The influence on gallbladder function was studied in two series of experiments (V) It was then found that the net water absorption from the gallbladder was reduced to one third of the basal value and a contraction corresponding to 22 per cent of the resting gallbladder volume was estimated Bile flow almost doubled and a pancreatic secretion of 4 ml/h was induced.

Installation of acid into the duodenum causes release of both secretin and CCK (Berry and Flower 1971 Schaffalitzky de Muckadell et al. 1977) Therefore one might feel tempted to assume that the reduced net water transport is entirely explained by a release of secretin and that the contraction is due to a release of CCK. However it is difficult to compare the complex situation when infusing acid by studies using i v infusion of isolated hormones since both hormone-hormonal and neurohumoral interactions are known to exist. These complex relationships are exemplified by the divergent results seen in different studies of gastric acid secretion during infusion of acid in the intestine (Wormsley 1971 Ward and Bloom 1975) The gallbladder response seen in the present study could entirely be explained by the influence of secretin and CCK. However several other hormones with known or probable effects on the gallbladder may be involved. Until the effects of the autonomic nervous system and other gastrointestinal hormones and their relationship on gallbladder function are known, it is impossible to fully elucidate which processes are involved.

GENERAL IMPLICATIONS

Physiological regulation of the gallbladder concentrating function?

Two observations published fortyfive years ago suggested that the concentrating function of the gallbladder had physiological variations (see page 9). In 1968 Diamond suggested that hormones and autonomic nerves were involved. During the last decade our knowledge in the field of the gastrointestinal peptides or hormones has vastly increased. In the present study it was found that secretin and VIP strongly influence the net absorption of water and electrolytes from the gallbladder. Further it was found that acid infusion into the duodenum also influences on the concentrating function of the gallbladder. These findings suggest that there is a physiological regulation. It could be argued that supraphysiological doses of the peptides were used and that the introduction of non-buffered hydrochloric acid into the duodenum is not a physiological procedure. Without studying the gallbladder in the conscious animal in different physiologic situations it is impossible to prove that there really is a regulation of its concentrating function. However, the prerequisites for regulation are accessible as secretin and VIP are available in the gastrointestinal tract and VIP containing nerve fibres exist topographically near the mucosal cells of the gallbladder.

The concentrating function of the gallbladder and the enterohepatic circulation of bile acids

As regards its organic contents the gallbladder can concentrate the hepatic bile entering its lumen tenfold. Fluctuation in the concentrating mechanism thus can vary the contents of for example bile acids in the gallbladder by a factor of ten. A pooling of bile acids in the gallbladder will decrease the rate of bile acids circulating enterohepatically whereas a mobilization of bile acids from the gallbladder will increase this rate. The absorptive state in the gallbladder will heavily influence the localization of the pool of bile acids, and thereby bile acid secretion from the liver. This is of pathophysiological interest since it has been shown both in man and mammals that the degree of cholesterol saturation of hepatic bile is inversely related to the rate of bile acid secretion (Scherstén et al. 1971, Wheeler and King 1972). A reduction of the net water absorption in the gallbladder by the influence of for example secretin or VIP decreases the possibility for the gallbladder to pool bile acids. This means that a greater fraction of the bile acid pool circulates enterohepatically maintaining a higher biliary bile acid generation rate, which in turn counteracts the tendency for cholesterol saturation.

Secretion in the gallbladder

The present studies are the first to demonstrate that the gallbladder containing its normal contents *in vivo* possesses the ability to secrete water and electrolytes into its lumen Attention to this function of the gallbladder has been drawn by Wood et al (1977) Schafer et al. (1969) reported from *in vivo* experiments in dogs that crude Vibrio Cholerae extracts introduced into a saline solution in the gallbladder lumen reversed the net water transport from an absorption to a secretion More recently secretin VIP prostaglandins and choleratoxin have been shown to induce a secretion in the isolated guinea pig gall bladder (Morton et al. 1977 Heintze et al. 1976 Saverymuttu et al 1977).

A VIP induced secretion and thereby a dilution of the gallbladder contents might be of importance for an efficient evacuation of the gallbladder contents after a meal. It might also be of importance for the elimination of precipitation nuclei for cholesterol in the gallbladder lumen.

VIP and the WDHA-syndrome

Raised plasma levels of VIP have been found in patients with the WDHA-syndrome and it has been suggested that VIP is a mediator substance in this syndrome (Said et al. 1976 Morton et al 1977) Beside the classical symptoms watery diarrhoea, hypokalaemia and achlorhydria this syndrome often includes a large atonic gallbladder containing dilute bile with raised concentrations of chloride and bicarbonate ions (Zollinger et al. 1968). The result of the present experimental studies in the cat demonstrates a relaxation of the gallbladder and a net water secretion into its lumen in response to VIP (III) These findings and the pattern of electrolyte secretion into the gallbladder lumen well agree with the supposed function of the gallbladder in the WDHA-syndrome This further corroborates the suggestion that VIP may be a mediator substance in this clinical syndrome

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Morphological and functional characteristics of the ageing skeletal muscle in man

A cross-sectional study

By
LARS LARSSON

STOCKHOLM 1978

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From the Department of Physiology, Karolinska institutet, Stockholm, Sweden

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CONTENTS

The present thesis is based on the following papers which will be referred to in the text by their Roman numerals In addition some hitherto unpublished results will be presented.

I Larsson L. B. Sjödin and J Karlsson Histochemical and biochemical changes in human skeletal muscle with age in sedentary males age 22-65 years Acta physiol scand. 1978 103 31-39

II Örlander J K.-H Kiessling L. Larsson J Karlsson and A. Aniansson. Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men. Acta physiol scand. 1978 In press

III Larsson L. G Grimby and J Karlsson. Muscle strength and speed of contraction in relation to age and muscle morphology J Appl Physiol Accepted for publication

IV Larsson L. and J Karlsson Isometric and dynamic endurance as a function of age and skeletal muscle characteristics Acta physiol scand. 1978 In press

V Larsson L. J H T Viitasalo and P V Komi Changes in reflex time and EMG signal characteristics in the ageing quadriceps muscle. Acta physiol scand. 1978 Accepted for publication

This paper is a part of a collaboration between two will also be a part in J Örlander s thesis

INTRODUCTION

During ageing changes take place in all organs although the time course of these changes is usually different for different organs or functional systems The atrophy of skin and muscles together with slowness and impairment of movements are probably the most striking features of old age The decline in strength and fine co-ordination and the increase in reaction time during ageing begin to manifest themselves during man s so called active period of life (Ufland 1933 Birren 1952 Burke *et al.* 1953 Asmussen and Heebøll-Nielsen 1961) Sporadic scientific interest has been focused on these alterations since Quetelet s (1836) pioneering study more than a century ago demonstrating a decline in maximum strength with age from approximately 25 years of age A better maintenance in maximum strength with increasing age has been reported in more recent studies i e maximum strength showed a small or non-existent decline from the twenties to the forties and then an accelerated decline with increasing age (Rejs 1971 Collumbine *et al.* 1950 Shephard 1969) Simonson (1964) assumed that this was due to the improvement of living conditions in general and suggested that further improvement might delay the process of ageing even more.

The major causes of the decline in motor performance in old age appear to be changes in the neuromuscular system, especially muscle atrophy even if other mechanisms have also been suggested to be responsible for this decline such as skeletal disorders (e.g osteoarthrosis) altered psychophysiological behaviour etc. (Gutmann and Hanzlíková 1972)

The atrophy seen in senile muscle has been suggested based on animal studies to represent a specific type of muscle atrophy although in many ways resembling the atrophy seen after denervation (Gutmann *et al.* 1971) For instance; muscle fibre atrophy decreased number of muscle fibres proliferation of the T-tubular and sarcoplasmic reticulum systems increased number of nuclei increased DNA concentration, and similar metabolic profiles have been reported in both the senile and denervated muscle (Gutmann and Hanzlíková 1966 1972/73) Alterations in the end-plate structures have also been reported in muscle resembling the picture seen in early stages of denervation number of synaptic vesicles accumulation and agglutination of and Hanzlíková 1972) However there is no electrophysiological denervation in the old muscle and there is a decrease in the fibres before there is a decrease in the number of nerve 1971)

It seems reasonable to assume that atrophy muscle especially reflects an altered

and Hanzlíková (1972) proposed that the senile muscle atrophy combines features of both a decline in nerve-impulse activity related to progressive disuse and a specific long-term neurotrophic disturbance resembling denervation in some functional aspects (functional denervation) which act relatively independently This emphasizes the importance of both exogenous (environmental) and endogenous (genetic) factors for ageing processes in the neuromuscular system.

Motor functions are known to be especially affected by environmental factors such as nutrition and physical activity Both cachexia and disuse cause muscle wasting with selective atrophy of type II (fast twitch) muscle fibres a type of muscle wasting also reported in old age (Engel 1970 Tomonaga 1977)

Increasing attention has been focused on endogenous changes of DNA and RNA in cells of ageing organisms which might affect the life span of the cells The changes seen in protein metabolism in old age have been suggested to be due to decreased RNA concentration and a disturbance of the transcription or translation process (Drahota and Gutmann 1961 Gutmann and Hanzlíková 1972) However in this field there are many unanswered questions as well as conflicting opinions and at this stage it can only be speculated upon whether the decline in muscular performance during ageing is mediated via a gene-directed process

Most of the results discussed above concerning senile muscle atrophy are based on animal studies However human muscle fibre types differ in many respects from those of cat and rat (Burke and Edgerton 1975) and this must be taken into account when comparing the information available from animal and human studies

The aims of the present investigations were to study basic characteristics morphological and functional of skeletal muscle in man at different ages An attempt was also made to correlate alterations in functional characteristics h the observed changes in muscle morphology

SUBJECTS

For study I and II muscle samples were taken from 55 healthy males 22-65 years of age who volunteered for the studies These men were all white-collar workers and were employees of the same insurance company Most of them were included in studies III (n = 51) IV (n = 50) and a smaller number in study V (n = 15) In study III a group of 29 10-19-year-old boys was also included. All these volunteers 10-65 years of age had a low level of physical activity i e they belonged to group I-II according to Saltin and Grimby s classification (1968) of occupational and spare time physical activity Study III also included a 70-year-

old group randomly selected from the population of Gothenburg (Rinder *et al.* 1975) which had a heterogenous physical activity background as they represented a random sample In study II 6 high school students aged 16-18 and 7 pensioners aged 66-76 were included the subjects in both these groups had various physical activity backgrounds Each subject was informed about the procedure and risks involved in the experiments before he agreed to participate and was further told that he was free to leave the study at any time.

METHODS

Anthropometric measurements (I V)

Measurements of height, body weight skeletal width skinfold thickness and thigh circumference were obtained for each subject. The skeletal measurements obtained were the bistyloid radioulnar and femoral condylar width. Skinfolds were measured at two locations at the knee (just above patella) and at the abdomen (at one third of the distance from the umbilicus to the spina iliaca anterior superior) From these values body fat weight skeletal weight and fat free soft tissue weight (FFB) were calculated (von Döbeln 1964) The circumference of the thigh was taken in a horizontal plane just under the gluteal furrow

Height and body weight in the 20-29- 30-39- to the 70-year-old group were compared with the corresponding age groups of a larger (n = 749) Swedish male population examined in these respects (Levin personal communication) Except for the 50-59-year-old group where height was found to be higher ($p < 0.05$) in the present subjects no significant differences were seen between the corresponding age groups.

Muscle biopsies

Muscle biopsy samples were taken from the middle portion of the resting quadriceps femoris muscle (m. vastus lateralis) using the needle biopsy technique (Bergström 1962) The vastus lateralis muscle is a major contributor to the force generated during knee extensions (see Thorstensson 1976) and is located at a site convenient for muscle biopsy sampling The choice of the quadriceps muscle had an additional advantage because it has been shown that ageing processes such as decline in maximum strength and muscle atrophy occur relatively early in this muscle (Charles 1964 Serratrice *et al.* 1968 Tomlinson *et al.* 1969)

Histochemical methods (I-V)

The muscle samples for fibre typing and area determination were trimmed, mounted in an embedding medium (Ames TM O C.T compound) frozen in liquid-nitrogen-cooled-isopentane and stored at -80°C until analysis

The myofibrillar ATPase method (Gomori 1941 Padykula and Herman 1955) was used for muscle fibre classification The reactions were carried out at pH 9 4 following alkaline preincubation (pH 10 3) By this procedure fibre classification can be made into type I (slow twitch) and type II (fast twitch) (Engel 1962) The type II fibres were subclassified into type IIA, IIB and IIC using preincubations at pH 10 3 4 6 and 4 3 (Brooke and Kaiser 1970 Dubowitz and Brooke 1973) The number of fibres of each fibre type was counted from a photomicrograph of the stained cross-sections and the relative proportion (per cent) of type I and II (A, B C) fibres was determined. The methodological error (coefficient of variance) of fibre type distribution (per cent) determinations has been reported as approximately 5-10% (Piehl 1974 Thorstensson 1976)

The average cross-sectional areas of the main fibre types (type I and II) were determined from serial cross-sections of each biopsy The fibres were identified from the ATPase staining while the areas were determined from the NADH tetrazolium reductase staining (Novikoff *et al.* 1961) in order to avoid shrinkage and mechanical artifacts caused by dehydration and rinsing

In study I the fibre areas were determined according to the method described by Thorstensson (1976) A photomicrograph of a NADH-stained section was made then selecting an area where the cut appeared to be well oriented at right angles to the long axis of the fibres areas representing the cross-sections of the ten largest fibres of each type were cut out from the photopaper pooled and weighed. By weighing the photopaper with known area an averaged area could be calculated for each fibre type The type II/I fibre area ratio together with muscle fibre type distribution were used in the computation of relative muscle fibre area (Gollnick *et al.* 1972) Determinations on biopsies obtained from the same muscle on two different occasions were found to have a coefficient of variance of 16-17% for the absolute areas of type I and type II fibres and 9% for the type II/I fibre area ratio (Thorstensson 1976) The wide variation in absolute values were suggested to be due to differences in the degree of contraction caused by the sampling procedure

Estimates of fibre areas obtained with the method described above is likely to be higher than the average fibre area as only the ten <u>largest</u> cross-sections are measured Measurement of all fibres disregarding size may give too low an estimate if some cross-sections are small due to their being

from e.g narrowing ends of fibres However measurement of all fibres should give an estimate with higher precision (as it is calculated from more observations) which in many cases is more important than the degree of bias For this reason cross-sectional areas were also calculated from the lesser fibre diameter assuming each fibre to have a circular *cross-section of diameter equal to the* lesser fibre diameter (Dubowitz and Brooke 1973) This allows a larger number of muscle fibre areas to be measured per biopsy and using this method an average of 200 $\pm$ 11 fibre diameters were measured per subject The values obtained according to Thorstensson (1976) were on average 2 11 $\pm$ 0 07 times higher than those obtained from the "lesser fibre diameter" method but the values correlated significantly ($r = 0.70$–0.80; $p < 0.001$) The higher precision of estimates calculated from the "lesser fibre diameter" method is indicated by the fact that they correlated better with e g age and strength (III IV) than estimates calculated according to Thorstensson (1976)

Biochemical methods (I II IV)

Muscle biopsies for enzyme activity analyses were quickly frozen in liquid nitrogen and stored in a freezer at $-80^{\circ}C$ for subsequent analyses The muscle samples were weighed in a cryostat (see Karlsson 1971) and homogenized by sonification and then diluted. The activities of Mg^{2+} stimulated ATPase (E.C. 3 6 1 4) myokinase (MK) (E.C. 2 7 4 3) and lactate dehydrogenase (LDH) (E.C. 1.1.1.27) were determined by fluorometric means using NAD-NADP coupled reactions according to Lowry and Passonneau (1972) (I IV) LDH activities were determined both for the oxidation of lactate and the reduction of pyruvate. The LDH isozyme pattern was determined with an electrophoretic method according to Dietz and Lubrano (1967) and was quantified by means of densitometric scanning (Penney *et al.* 1974 ad modum Sjödin 1976)

In study II the muscle samples were homogenized in a Potter-Elvehjem glass homogenizer and assays were performed on the day of biopsy The activities of phosphofructokinase (PFK) (E.C. 2 7 1.11) LDH 3-hydroxyacyl-CoA dehydrogenase (HAD) (E.C. 1 1 1 35) citrate synthase (CS) (E C. 4 1 3 7) and cytochrome oxidase (cytox) (E.C. 1 9 3 1) were determined. PFK activity was determined by the method of Shonk and Boxer (1964) LDH and HAD according to Bass *et al.* (1969) CS as described by Srere (1969) and cytox according to Wharton *et al.* (1969) Muscle protein content was determined with the Folin reaction as modified by Lowry *et al.* (1951)

Strength measurements

Maximum isometric and dynamic strengths were measured in the left knee-extensor muscles using an isokinetic dynamometer (Cybex II Lumex Inc. New York) The subjects were seated in an adjustable chair with support for the back shoulders and hips The hip angle was fixed at /2 rad (90 degrees) and the lower leg moved the lever of the dynamometer The lever was kept at a constant length and attached to the tibia. The centre of the dynamometer s axis of rotation was aligned with the anatomical axis of rotation i e the knee joint. The angular velocity was controlled at a pre-set level by an internal resistance which accomodates to the muscular force applied (Hislop and Perrine 1967 Thistle *et al.* 1967 Perrine 1968) The angular movement of the knee joint was from 0 55 π to 0 rad (100 and 0 degrees i e full knee extension) Isometric strength was measured at selected knee angles (π /6 π /3 and π /2 rad or 30 60 and 90 degrees) whereas the dynamic strength was recorded over the whole range of motion at different angular velocities The velocities studied were π /6 π /3 2 π /3 and π rad x s^{-1} (30 60 120 and 180 degrees x s^{-1}) Two attempts were allowed at each velocity or knee angle and the highest value was noted. The produced torque was calculated as the force times the length of the lever and unit given is Nm. The measurements were made in sequence from slow to fast speeds with 30 s recovery between each contraction The following sequence was used: π /6 rad x s^{-1} π /6 rad π /3 rad x s^{-1} π /3 rad 2 π /3 rad x s^{-1} π /2 rad, and π rad x s^{-1} High precision and accuracy for the torque registrations have earlier been reported (Moffroid *et al.* 1969 Thorstensson *et al.* 1976) The influence of muscular fatigue due to the testing procedure may considered to be negligible according to Thorstensson (1976)

Muscular endurance was assessed in both isometric and dynamic terms Maximum isometric strength (MIS) and isometric endurance respectively were determined for both legs simultaneously (Karlsson and Ollander 1972) by recording the force exerted when the subjects pressed their feet against a stiff bar equipped with a force transducer MIS was taken as the highest force value obtained during a series of five contractions After three minutes rest isometric endurance time was recorded measured as the maximum time during which a tension level of 50% of MIS could be maintained.

Dynamic endurance was measured as the ability to maintain tension output during repeated maximal dynamic contractions performed on the isokinetic dynamometer The angular velocity was pre-set at π rad x s^{-1} (180 degrees x s^{-1}) with the subject seated as described above Dynamic endurance was calculated from

measuring 50 maximal contractions and determining the absolute (Nm) or relative (%) decline in peak torque from the mean of the three initial contractions to the mean of the three final contractions i e subjects with a low force decline had a high dynamic endurance and *vice versa*. The methodological error (coefficient of variation) for this measurement has been reported to be 3 2% (Thorstensson and Karlsson 1976)

Maximum knee extension velocity (MEV) was assessed by means of a much lighter level compared to that used for strength measurements Sitting in the same experimental chair used for strength measurements the subjects were instructed to extend the leg as quickly as possible starting from a knee angle of π /2 rad (90 degrees) The subjects performed five test extensions to become familiar with the apparatus and the procedure Then they made five subsequent extensions with a 20 rest between each The time required to extend from 0 42 to 0 08 π rad (75 to 15 degrees) was measured and the highest angular velocity attained during the five trials was used as a value for MEV The coefficient of variation for MEV measurements has been reported to be 5 2% (Thorstensson 1976)

EMG recordings

The electrical activity of m. rectus femoris was picked up using surface electrodes (E & M Instruments Co) during isometric endurance tests (maximum performance time at 50% MIS) The electrodes were placed near the middle on a line from spina iliaca anterior superior to the patella. The EMG signals were amplified with a Tektronix RM 122 low-level preamplifier and subsequently stored in analogue form on a Philips Analog 7 tape recorder The recording speed was 380 mm s $^{-1}$ The EMG signals were analysed for their mean power frequency (MPF) according to Kwatny *et al.* (1970) and relative portions of selected bandwidths (24-48 Hz 56-96 Hz 104-136 Hz and 144-400 Hz) as described earlier (e g Viitasalo and Komi 1975 Komi and Viitasalo 1976)

Patellar reflex measurements

The patellar reflex time was recorded with a set-up (Komi *et al* 1973) in principle similar to the one described by Tipton and Karpovich (1966) The subject was sitting in an experimental chair with /2 rad (90 degrees) knee and hip angle. The quadriceps tendon was tapped by an electrical hammer with adjustable arm length The force impact on the ligamentum patellae was maintained constant by allowing the hammer to move through an area of π /2 rad (90 degrees) and using a constant hammer arm length (40 cm) On contact with the tendon a

switch was closed on the hammer producing an electrical signal. Another signal was obtained at the moment the foot started to move forwards by means of a microswitch located at the heel. The electrical activity of m rectus femoris was picked up using surface electrodes located at the same position as during the EMG recordings (see above)

Signals from the switches on the hammer and behind the heel were stored together with the EMG signals on magnetic tape (Philips Analog 7) using a recording speed of 380 mm x s^{-1} The tape was played back at 24 mm x s^{-1} and the signals were displayed on a graph recorder The time from the hammer s contact with the quadriceps tendon to the beginning of the heel movement was defined as total reflex time (TRT) This was further divided into reflex latency (LAT): the time from the tendon was tapped to the first discernible EMG signal; and reflex motor time (MT): the time from the first EMG signal to the beginning of leg movement.

A high precision was found in the patellar reflex time measurements i e the variation from five subsequent observations on each subject revealed a small variation in measuring the components of the patellar reflex time (S D = 0 4-6 5) compared with the variation between subjects (S D = 1 5-43 5)

Statistical methods

Arithmetic means standard error of the means (S E) and linear correlation (r) were calculated from individual values according to Snedecor and Cochran (1967) One way analysis of variance was used to test differences between the age groups (Scheffe s method see Snedecor and Cochran 1967)

In order to compare changes with age in isometric strength dynamic strength and HEV curvilinear regressions were applied to individual data. Thus (1) Strength = $a + b_1x + b_2x^2 + \quad + b_nx^n$ where a and b are constants and x is the age of the individual From this it was found that (2) strenght = $a + b_1x + b_2x^2 + b_3x^3$ made a good fit and made comparisons possible between the different variables studied.

Stepwise linear multiple regression analysis was applied according to Nie *et al.* (1975) with strength and HEV as dependent variables and anthropometric and histochemical data together with subject s age as independent variables In each step the variable added to the equation was the one that made the greatest reduction in estimated residual variance The last step (= equation) given in Results is the step with minimum residual variance

To evaluate the dependence of strength and HEV on the histochemical data compared with the dependence on age a hierarchical model was applied The

independent variables were then entered in an order specified by the investigators i e. the histochemical variables were entered first in the equation and in the last step age was entered. The specific order of the histochemical variables were determined from stepwise multiple regression analyses where age was excluded.

RESULTS AND COMMENTS

Muscle biopsy findings

Histochemical characteristics (I III IV) Fibre type distribution data for the 55 *subjects 22 to 65 years of age displayed a linear decrease (r = -0 46; p 0 001)* in the proportion of type II fibres with increasing age Thus the relative proportion of type II fibres was 59 ± 4% in the 20-29-year-old group compared with 45 ± 4% in the 60-65-year-old group No significant differences with age in fibre type distribution within the type II fibre population were observed (Table I)

In study I the average cross-sectional area of type II fibres was observed to decrease (r = -0 36; p < 0 01) with age while no significant linear change was seen in type I fibre area resulting in a decreased (r = -0 33; p < 0 02) type II/I fibre area ratio from 1 24 ± 0 05 (20-29 yrs) to 0 96 ± 0 05 (60-65 yrs) Consequently the relative type II fibre area decreased (r = -0 55; p 0 001) from 64 ± 4% (20-29 yrs) to 44 ± 4% (60-65 yrs)

In study III and IV the fibre areas were determined according to the lesser fibre diameter" method By this method compared with the method used in study I smaller average fibre areas were found. However similar age-

Table I. Distribution of fibre types within the type II fibre population.

Age group (yrs)	n	% of type II fibres		
		IIA	*IIB*	*IIC*
20-29	7	59±2	35±3	6±1
30-39	11	61±4	34±3	5±2
40-49	7	70±7	27±7	3±1
50-59	9	60±6	37±5	3±1
60-65	7	62±7	32±8	6±2

Table II Fibre areas and fibre area ratios of the different age groups according to "lesser fibre diameter" method.

Age group (yrs)	Average age (yrs)	n	Type I area (100 x μm²)	Type II area (100 x μm²)	Area ratio Type II/I
20–29	26	11	29 5±2 5	36 6±2 2	1 28±0 07
30–39	36	12	29 2±1 5	36 7±2 9	1 27±0 09
40–49	43	10	31 9±1 9	32 6±2 4	1.03±0 06
50–59	55	12	28 8±1 6	28 0±1 2	0 99±0 05
60–65	62	10	22 6±2 4	21 2±1 7	0 99±0 09

trends were seen when using the "lesser fibre diameter" method (Table II) i e the type II fibre area declined with age ($r = -0\ 60$; $p < 0\ 001$) as did type II/I fibre area ratio ($r = -0\ 47$; $p\ \ 0\ 001$) whereas no statistically significant linear change was seen in the type I fibre area. The stronger correlations with age using the "lesser fibre diameter" method, compared with the method used in study I probably reflects the higher precision of this method as argued in the method section The distribution of the fibre diameters for the 20-29- and 60-65-year-old groups is shown in Fig 1 demonstrating a displacement to smaller diameters of the type II fibres in the oldest age group while almost no difference was seen in the distribution pattern of type I fibre diameters

Comments So far no systematic studies on skeletal muscle histochemistry in healthy men 20 to 65 years of age are available. Studies by Gollnick *et al* (1972) Kiessling *et al* (1974) and Saltin *et al.* (1976) showed fibre type distribution data in untrained young and middle-aged men which compare well to the present data (Fig 2) Hedberg and Jansson (1976) reported an average of 46 per cent type II fibres in 69 16-year-old boys Their subjects were however of a heterogenous physical activity background, not in the same age-span and differing in physical activity level and so are not comparable with the subjects presented here Ängquist (1978) commented a decreased incidence of type II fibres with age (43-69 yrs n = 20) in m. tibialis anterior in the symptomatic as well asymptomatic leg in patients with unilateral intermittent claudication supporting the findings seen here

Type II fibres larger than type I fibres have been shown in young physically untrained males (Gollnick *et al* 1972 Saltin *et al* 1976) The present data are in agreement with this Bass *et al* (1975) showed an atrophy

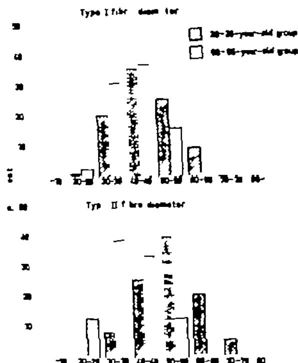


Fig 1 The percentage distribution f muscle fibre diameters of both fibre types in the 20-29- and 60-69-year-old groups Arbitrary units are used for muscle fibre diameters

of the type II fibres in the senile rat muscle (m. ext dig long) whereas no change was seen in type I fibre area. In a recently published study on human subjects (79 patients age 60-90 yrs) similar changes were observed demonstrating a linear decrease ($p < 0.01$) of the type II/I fibre diameter ratio with age from approximately 1.0 (60-70 yrs) to 0 6 (80-90 yrs) (Tomonaga 1977) Aniansson et al.

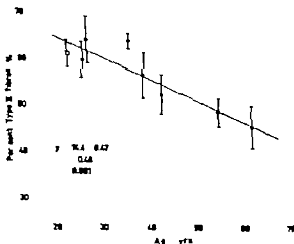


Fig 2 Per cent type II fibres versus ag Mean values ± S E are shown along with the regression line Regression line is calculated from indi idual alues Data from Gollnick et al (1972 O) Kiessling t l (1974,Δ) Saltin t l (1976 □) ar also plotted

(1978) reported a type II/I fibre diameter ratio corresponding to 0 95 in the quadriceps muscle of 70-year-old men (fibre area ratio 0 91) The present data when expressed as type II/I fibre diameter ratio demonstrating a decrease from 1 13 (20-29 yrs) to 0 99 (60-65 yrs) are in good agreement with these findings

Biochemical characteristics (I II) Only small changes were seen with increasing age (20-65 yrs) in the different muscle enzyme activities studied (HAD PFK LDH CS cytox, MK and Mg^{2+} stimulated ATPase) However HAD activity increased slightly ($r = 0.28$; $p < 0.05$) with age and tendencies to increased CS and cytox activities were seen. A shift in LDH isozyme pattern was observed with age towards a decrease in per cent muscle-specific isozymes (M-LDH) ($r = -0.39$; $p < 0.01$) This took place by means of a decrease ($r = -0.37$; $p < 0.01$) in M-LDH activity with age while no change was seen in heart-specific isozyme activities M-LDH HAD CS and cytox correlated significantly ($r = 0.34$-0.39; $p < 0.01$) with per cent type II fibres demonstrating an increased M-LDH activity and a decreased HAD CS and cytox activity with an increasing proportion of type II fibres

Comments The increased HAD activity with age and the similar trends for CS and cytox indicate a tendency towards increased oxidative capacity in old muscle This is further supported by the decreased activity of the anaerobic M-LDH while the aerobic" H-LDH was unaltered or slightly increased with age A similar age-change in the proportion of LDH isozymes has been observed in the rat heart muscle (Kanungo and Singh 1965) The most plausible explanation to the alterations in enzyme activities with age is the parallel increase in per cent type I fibres since type I fibres have a greater oxidative potential than type II (e g Essén *et al* 1975)

The tendencies towards increased activities of oxidative enzymes with age were not, however accompanied by a corresponding increase in mitochondrial volume on the contrary the mitochondrial volume decreased with age (II) This indicates an increased oxidative capacity per unit mitochondrial volume which might be achieved by an increase in the respiratory assemblies on the inner mitochondrial membranes (cf Kiessling *et al* 1974 1975)

Muscular performance

Maximum isometric and dynamic strengths (III) Isometric and dynamic streghts (at all velocities studied) increased steeply from the 10-14- to the 20-29-year-old group remained unchanged to the 40-49-year-old group and then decreased with increasing age (10-69 yrs n = 89) The decline in strength in old age was not however accompanied by any measurable external changes in muscle mass (i e thigh circumference) According to curve fittings isometric and dynamic

strengths reached their peak values in the same ages This was also true when strength was expressed per kg body weight or FFS High correlations (r = 0 86–0 89 n = 89) were found between isometric strength and dynamic strengths as well as between dynamic strengths at different speeds of contraction (r = 0 86–0 95 n = 89)

Significant linear correlations (r = 0 44–0 54; p < 0 01–0 001) were found between strength isometric as well as dynamic and type II fibre area. Weaker although statistically significant correlations were seen between strength and type I fibre area (r = 0 28–0 39; p < 0 05–0 01)

Comments: In order to study the influence of muscle fibre area on strength compared with other age factors multifactor analyses were applied When the influence of type II fibre area on strength was controlled age still had a significant partial correlation with the strength decline in old age This seems to suggest that other factors together with muscle fibre atrophy are responsible for the age-related strength decline

Maximum knee extension velocity (MEV) (III) MEV followed an age-pattern similar to that found for strength with age although the changes in MEV with age were less pronounced. The mean values for strengths increased by 120–140% between the 10-14- and the 20–29-year-old groups and the values for the 60-69-year-old group were 26–38% below the mean values found for the 20–29-year-old group The corresponding values for MEV were +13 and -7% respectively

Per cent type IIB fibres was the only histochemical variable found to correlate significantly to MEV in simple as well as multiple regression analysis.

Isometric and dynamic endurance and EMG (IV V) Neither isometric nor dynamic endurance changed significantly with age although a slight increase was seen. Both isometric and dynamic endurance were measured in relation to maximum strength thereby compensating for individual and age differences in strength. However absolute maximum strength decreased with age indicating a reduced isometric and dynamic endurance capacity when expressed in absolute terms (Fig 3)

Isometric endurance correlated poorly with the muscle fibre characteristics studied. Examination of the individual EMG frequency spectrum bandwidths during sustained isometric contractions showed that the proportion of the lowest bandwidth (24-48 Hz) increased more in the youngest age group (25–30 yrs n = 4) compared with the older groups (40–45 yrs n = 6 and 60–65 yrs n = 5) (p < 0 05–0 01) This relation was also seen in regression analyses (r = -0 66; p < 0 01) The increase in the lowest bandwidth also correlated to the

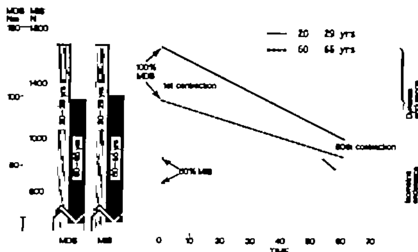


Fig 3 Maximum dynamic (MDS) and isometric trength (MIS) are shown for the 20-29- and 60-65-year-old groups Dynami endurance measured as the ability to perform 50 repeti tive maximal dynami contractions and isometri endurance mesured as the maximum time during which tension level of 50% MIS could be maintained are also shown for both age groups

relative cross-sectional area of type II fibres (r = 0 73; p < 0 01)

The absolute (Nm) and relative (%) force decline during 50 repeti tive dynamic contractions were used as an expression for dynamic endurance i.e subjects with low force decline had a high dynamic endurance and *vice versa* The absolute force decline increased with increasing relative type II fibre area and increasing H-LDH activity (r = 0 34-0 51; p 0 05-0 01) The relative force decline increased with an increasing proportion of type II fibres as well as an increasing relative type II fibre area (r = 0 38-0 44; p < 0 05-0 01) Both the absolute and relative force decline decreased with an increasing cytox activity (r = -0 45 -0 46; p < 0 01)

Patellar reflex measurements (V) The motor time component (MT) of the patellar reflex was found to be longer (p < 0 05) in the 60-65-year-old group (n = 5) compared with the 25-30- (n = 4) and the 40-50-year-old group (n = 6) according to one way analysis of variance This was also true when motor time was related to age in linear regression analysis (r = 0 61; p 0 02) The reflex latency (LAT) did not, on the other hand differ significantly between the groups although a slightly lower value was seen in the youngest group Consequently the total reflex time (TRT) showed an increase with age (r = 0 60; p < 0 02)

The LAT tended to increase with increasing upper leg length ($r = 0.50$; $p < 0.1$). To compensate for anatomical variations between subjects, a crude estimate of "nerve conduction velocity" was obtained by dividing LAT by 1.5 upper leg length. The nerve conduction velocity calculated was found to decrease with increasing age ($r = -0.56$; $p < 0.05$), demonstrating a 10% decrease in absolute figures between the 25–30- and the 60–65-year-old group. A significant correlation ($r = 0.59$; $p < 0.05$) was seen between per cent type II fibres and nerve conduction velocity.

Comments: The reduction in nerve conduction velocity observed here is in accordance with previous studies (Wagman and Lesse 1952, Norris *et al.* 1953) who also found a 10% decline between 20–29 and 60–69 years of age. The correlation between per cent type II fibres and nerve conduction velocity indicates that the decline in per cent type II fibres with age is somehow related to the decline in nerve conduction velocity.

GENERAL DISCUSSION

Possible mechanisms underlying the alterations seen in muscle composition with age

The question regarding possible mechanisms underlying the alterations in muscle morphology observed with advancing age immediately arose. Because the total number of muscle fibres in the muscle at different ages is not known, no conclusive answer to this question can be given. It seems reasonable, however, that one or more of the following factors might, at least in part, explain the altered muscle morphology with increasing age: 1) a systematic error in the sampling of subjects, 2) a transformation of type II fibres to type I caused by changing properties of the motoneurones or collateral sprouting, 3) an altered level of physical activity, and 4) a slow denervation process and a progressive preferential loss of type II fibres. These factors may partly interact with each other and therefore no absolute dividing lines can be drawn between them.

1. Sampling errors. Studies of ageing can be made either as a longitudinal or as a cross-sectional study. Neither of these approaches is free from sampling errors (cf. Bromley 1971). In the present papers a cross-sectional design was chosen because it can be completed without waiting a considerable time for alterations to appear in the measured parameters and since it is obviously impractical to study a population group for sixty years by a longitudinal design. The main disadvantage of the cross-sectional method is that it can never be used to evaluate

age changes in single individuals since it is nearly impossible to get the subjects in the different age groups to be comparable in all respects except age However by the cross-sectional method an estimation of average age trends can be made despite the fact that the different observations will be relatively widely scattered. Particular effort was made to obtain subjects that were as homogenous as possible as regards physical activity i e they all had a low level of physical activity

As pointed out above fibre type distribution data as well as fibre area ratios correspond well with previous observations in animals and man indicating that a systematic error in the sampling of subjects seems to be a less plausible reason for the age trends seen in muscle composition

2 Transformation of muscle fibre types An increase in the percentage of type I fibres has been observed during growth and maturation in animals as well as in man (Karpati and Engel 1967 Kugelberg 1976 Koens *et al.* 1978) This has been suggested to be due to a transformation of type II fibres to type I rather than degeneration of type II fibres since the total number of muscle fibres increased and the diameter of the type II fibres increased until they were transformed" (Karpati and Engel 1967) According to Kugelberg (1976) transformation being due to changing properties of the motoneurones i e. from phasic to tonic

Bass *et al* (1975) have shown that there is a clear trend of de-differentiation of the biochemical and histochemical pattern during ageing indicated by signs of type grouping a decreased total number of muscle fibres and muscle fibre atrophy This was shown both in the fast (m. ext dig long) and slow (m. soleus) rat muscle These data are different from the progressive development during growth and maturation in respect to functional biochemical and histochemical properties The type grouping observed (Bass *et al* 1975) was thought to be due to collateral sprouting caused by a progressive denervation-reinnervation process during ageing

Type grouping has also been reported in human senile muscle (Tomonaga 1977) Tomonaga showed however that the type grouping was less pronounced in the proximal muscles of the lower extremity An increased incidence of type IIC fibres has been reported during the denervation-reinnervation processes (cf Jansson and Kaijser 1977) In the subjects presented here no pronounced type grouping was observed and the per cent type IIC fibres was unaltered with age The denervation-reinnervation process via collateral sprouting therefore seems less plausible as being the only contributor to the histochemical findings presented here.

3 *Effect of physical activity and training* In the ageing muscle it is very difficult to distinguish between changes due to ageing *per se* and degenerative lesions due to impairment of cardiovascular or locomotor functions or to disuse. The subjects in our studies were by their own account and after physical examination found to be without locomotory defects or cardiovascular diseases. The subjects from whom muscle biopsies were taken (20–65 yrs n = 55) had an almost equal spare time and occupational physical activity level and were all in the "productive period" of their life It cannot be ruled out however that the subjects had adapted to a more sedentary way of living with age Irrespective of whether this adaptation is an endogenous or a sociological-anthropological phenomenon it must be regarded as a part of the overall ageing process in man with a significance for muscle tissue adaptation.

A preferential atrophy of type II muscle fibres has been reported after inactivity (Engel 1970) suggesting that the muscle atrophy seen in old age might be related to progressive disuse If maximal efforts were performed less often in old age requiring less intense nerve impulse activity and less activation of the high threshold (phasic) motor units this might very likely result in a preferential atrophy of type II fibres

A subsample of the present material (19 subjects age 22–65 yrs) took part in an intense muscular strength training program for 4 months (Larsson *et al.* to be published) When muscle biopsies were taken after the training period and compared with those obtained before the training it was observed that the reduction in type II fibre area in the old age subjects was no longer significant. This observation strengthens the likelihood that preferential type II fibre atrophy with increasing age might be related to a more sedentary way of living

4 *Functional denervation and atrophy* Gutmann and Hanzlíková (1972) stated that the morphological and metabolic characteristics of the senile muscle could not be solely explained by a reduced nerve impulse activity related to progressive disuse in old age This conclusion was based on the fact that the atrophy caused by disuse does not result in a decreased number of muscle fibres and the ultrastructural as well as metabolic changes reported in both senile and denervated muscle (Gutmann and Hanzlíková 1972) They therefore proposed that senile atrophy combines features of both disuse and functional denervation a specific long-term neurotrophic disturbance resembling denervation in some functional aspects They also proposed that a distinction between "healthy" and dead motoneurones is an oversimplification and that "sick motoneurones should be considered signifying a disturbance in neuromuscular contact. The senile motoneuron would

then serve as an important model for such a disturbance. According to McComas *et al.* (1971) sick motoneurones are characterized by an impaired neuromuscular transmission, an incapacity to innervate previously denervated muscle fibres via collateral sprouting and a slowing in impulse conduction velocity

Gasser (1944) found that the calibre spectrum of the dorsal and ventral root fibres appeared to shift to smaller diameters at advanced ages which he interpreted to be due to a selective atrophy and degeneration of the largest and fastest conducting nerve fibres These fibres mainly innervate the type II fibres (cf Burke and Edgerton 1975) The selective degeneration of the fastest conducting nerve fibres has been suggested (cf Sunderland 1968) to be one contributing factor to the decreased nerve conduction velocity during ageing (Wagman and Lesse 1952 Norris *et al* 1953) In support of these findings Campbell *et al* (1973) showed (electrophysiologically) a decreased number of functioning motor units in old age and that the remaining motor units tended to have relatively slow twitches

It thus seems that the altered fibre type distribution seen with increasing age is at least due in part to a slow loss of α-motoneurones innervating the type II fibres However it cannot be determined whether this is caused by an ageing process which preferentially involves the fast twitch units or whether it is the result of inactivity in the fast twitch units because of an altered physical activity pattern during ageing or both these

Muscular performance

1 Muscular strength In the pioneering study by Quetelet in 1836 isometric muscular strength was reported to decrease linearly with age from approximately 25 years More recent studies have however shown an improved maintenance in muscular strength during ageing (Reja 1921 Collumbine *et al* 1952) In the study by Shephard (1969) North American subjects were found to reach peak isometric strength at 18 years of age to maintain constant strength to 40–49 yrs and then gradually decline in strength performance. The present observations are in good agreement with these more recent data

To the author s knowledge there is no published information concerning standardized measurements of dynamic strength and its relation to age In parallel to the present study strength measurements have been obtained in physically active men national competitive oarsmen and former national competitive oarsmen still physically active in rowing (18–45 yrs) (Larsson, unpublished *observations)* In these subjects no significant differences were seen in isometric or dynamic strength in the knee-extensor muscles with increasing age This finding

thus seems to be in line with those of the main study as well as the findings quoted above indicating that there is little if any loss of muscular strength, isometric as well as dynamic between 20 and 45 years of age.

Muscular strength is proportional to the active tional area of the muscle which can be expressed as the total number of muscle fibres x average fibre area x per cent activated fibres Hence strength should be related to the total cross-sectional area of the muscle but the correlation need not be absolute (cf Astrand and Rodahl 1977)

A variety of data from the present investigation could be used to estimate the total ... onal area of the muscle for different ages Thigh circumference was measured for all subjects; this did not change with age. However this need not mean that the muscle area remains constant since it is known that there is a gradual replacement of muscle tissue by connective tissue and fat with advancing age as well as an increase in subcutaneous fat (Frantzell and Ingelmark 1951 Allen *et al* 1960) According to data from Häggmark *et al*. (1978) based on computorized tomography the histochemical variable which most closely correlates with muscle cross-sectional area is the average fibre area (% type I x type I area + % type II x type II area) This variable was computed for the subjects and was found to decrease with age. A significant correlation between strength and mean fibre area was found (r = 0 40–0 53) An equally strong correlation between strength and type II fibre area was also found (r = 0 43–0 54) This is not surprising since type I fibre area did not change with age.

Can the decrease in strength with age be explained by type II fibre atrophy alone? Cursory inspection of the data suggests that this is a possibility The mean value for strength decreased by 26-38% from the 20-29- to the 60-65-year-old group (isometric and dynamic strength at the highest velocity studied respectively) whereas mean type II fibre area decreased by 42% These figures may however be deceptive because there were relatively few subjects in each age group and there was considerable scatter of the values Secondly in multiple regression analysis it was found that when the independent influence of type II fibre area on strength was eliminated age still had a significant negative partial correlation with strength suggesting that other factors might contribute to the decline in strength.

The apparent discrepancy between the extent of strength decline and decrease in type II fibre area might be explained by the motor unit recruitment pattern.

Ikai *et al* (1967) found a 30% increase in force during electrical stimulation of muscle compared to the force exerted during maximal voluntary contractions indicating an inability to recruit all motor units under 1

conditions According to Secher *et al.* (1978) there is reason to believe that this phenomenon is especially pronounced in physically untrained subjects

Type II muscle fibres are known to be contained in motor units with high activation thresholds and type I muscle fibres in motor units with low activation thresholds representing a continuum of thresholds within each fibre type population (Burke 1968 Engel 1970 Close 1972 Gollnick *et al.* 1974) It seems reasonable to suggest that some of the high threshold motor units were never recruited in the type of maximal voluntary efforts studied here neither by the younger nor by the older subjects If this is true the type II fibre atrophy seen in old age need not reflect a concomitant decline in the active cross-sectional area of the muscle

A subsample of the present material (22-65 yrs n = 19) was involved in an intense muscular strength training program twice a week for four months (Larsson *et al* to be published) Similar trends in muscle characteristics (morphological and functional) with age were present in the subsample i e there was significant decline in both type II fibre area and strength (isometric and dynamic) with increasing age. Significant increases in maximum strength were found after the training period irrespective of the subjects age although strength still was found to decline significantly with increasing age On the other hand after the training period, type II fibre area in the older groups was not significantly lower than for the younger groups although there was a tendency towards a decrease with age This further emphasizes that factors other than muscle fibre atrophy also are responsible for the decline in strength in old age.

Maximum strength has been reported to be limited by psychologically induced inhibitions which can be reduced by a loud shout or a gun shot (Ikai and Steinhaus 1961) The motivation of the subject to perform maximal effort is another factor resulting in a methodological error even in standardized muscular strength measurements (cf Åstrand and Rodahl 1977) The possibility that the decline seen in strength in old age can be ascribed to an altered motivation is at present impossible to evaluate However this does not seem to be a plausible reason for the decline in strength in the old subjects especially since the ability to perform a sustained isometric contraction or repeated maximal dynamic knee extensions was unaltered in old age

As noted above the cross-sectional area of the muscle is not only related to fibre area but also to number of muscle fibres A pronounced decline in number of functioning motor units has been reported to occur after approximately 50 years of age in human skeletal muscle (Campbell *et al* 1973 Sica *et al* 1976) Since strength development followed a similar pattern with increasing age it

seems reasonable to suggest that the decline in strength in old age may be due both to fibre atrophy and to a decreased number of muscle fibres

2 Shortening velocity In the present study (III) maximum knee extension velocity (MEV) was found to decline slightly with increasing age Interestingly a statistically significant correlation was also seen between MEV and per cent type IIB fibres This is in accordance with Campbell *et al.* (1973) who proposed that the reduced speed of contraction reported in old age (Gutmann *et al.* 1971) *might be explained by the fact that most of the surviving motoneurones in old* age innervate muscle fibres of slow twitch type (type I) However the relative changes seen in MEV with age were less pronounced compared with changes seen in strength The findings presented here support the hypothesis that the maximum speed of contraction is in contrast to strength, mainly dependent on muscle quality rather than quantity

3 Muscular endurance Isometric and dynamic endurance in the knee-extensor muscles measured in relation to maximum strength, appeared to increase with age although not statistically significantly In two other studies similar tendencies towards increased endurance (isometric) were seen in old age in the hand-grip muscles (Petrofsky and Lind 1975a b Aniansson *et al.* 1978) Petrofsky and Lind suggested, on the basis of results from animal studies (Drahota and Gutmann 1963 Close 1964 Gutmann and Hanzlíková 1972) that the changes found in muscular endurance with age might be due to changing proportions of type I and type II fibres giving a higher proportion of type I fibres This hypothesis appears tenable since such a change in fibre proportions has been observed here

Frequency analysis of the EMG during long-lasting isometric muscle contractions showed an increase in the low-frequency region whereas the high-frequency signals decreased (Kogi and Hakamada 1962 Kaiser and Petersén 1963) *This altered frequency spectrum during fatigue has* been interpreted as being due to a decline in conduction velocity of the action potential along the muscle fibres (Lindström *et al.* 1970) The accumulation of metabolic by-products (primarily lactic acid) in the muscle fibres resulting in changed excitability of the cell membrane has been suggested to be the main cause of the decrease seen in conduction velocity (Mortimer *et al.* 1970) The increase in the low-frequency region was more pronounced in the young than in the old subjects during long-lasting isometric contraction indicating a less pronounced decrease in impulse conduction velocity in old age It may be suggested that this is primarily due to age-dependent changes in the muscle tissue i.e. decreased relative cross-sectional area of type II fibres since the lactate production has been shown to

be higher in type II than in type I fibres during long-lasting isometric contractions (Essén and Häggmark 1975)

Lactate accumulation has been proposed as an important limiting factor in the types of isometric and dynamic endurance tests used here (Hultén *et al.* 1975 Tesch *et al.* 1978) This suggestion is further supported by the results presented here which show an increased dynamic endurance with decreased H-LDH activity and increased cytox activity since a decreased H-LDH activity and an increased cytox activity might result in a smaller lactate accumulation via reduced lactate production and oxidation of pyruvate

The rate of perceived exertion (RPE) is another factor which influences the ability to perform prolonged work Borg and Linderholm (1967) reported that RPE at a given work intensity (bicycle ergometer exercise) was unaltered with increasing age in 61 lumber workers (27-63 yrs) whereas in a mixed group (18-79 yrs) RPE increased with age The dissimilarity between these two groups was interpreted to be due to the heterogenous physical activity background of the mixed group i e the older subjects had adapted to a lower physical activity level However the exertion measured during a submaximal bicycle ergometer work is most probably different from the type of exertion and the local muscular fatigue in the muscular endurance measurements used here The local muscular fatigue experienced during prolonged work with hand ergograph has been reported to be altered in old age (cf Frolkis *et al* 1976) i e in young persons there is a gradual decrease in working capacity whereas in old age the exhaustion is experienced first at an extreme level of muscular work The present tendencies towards an increased endurance in old age are thus in line with these data

4 Reflex measurements The method of fractionation of the total patellar reflex time used here separates the reflex in two discrete elements: first a neuronal component LAT (reflex latency) and second a peripheral component MT (motor time) (Hayes 1972) The present results indicate that the increased reflex time seen in the oldest group was primarily due to a rise in motor time i e the time taken for spread of excitation and development of sufficient tension within the muscle to overcome inertia and set the limb in motion. There are consequently different possible reasons which alone or together might be responsible for the increased MT with age

A slower and/or weaker muscle contraction in the older subjects would cause an increased latency before the lower leg starts to move because the rise of tension is less steep This might be caused by:

- a reduced motor nerve activity which in turn could be due to an impaired

transmission of the tendon tap to the muscle spindles a decreased sensitivity of the muscle spindles caused by the capsular thickening and the decreased number of intrafusal fibres reported in old age (Swash and Fox 1972) and/or a less efficient transmission to the α-motoneurones

- the histological and ultrastructural changes observed in the muscle tissue itself with age such as muscle fibre atrophy altered proportions of muscle fibre types proliferation and dilatation of the T-system and sarcoplasmic reticulum etc. (I Gutmann *et al.* 1971 Tomonaga 1977)

Increased joint stiffness reported in old age (Wright and Johns 1960 Long *et al.* 1968 Chapman *et al.* 1972) is another factor which might increase the MT since an increased stiffness in the knee-joint would lead to an increased opposing force for the contracting muscle thus leading to a prolongation of the time before movement in the knee-joint occurs

SUMMARY

1 Morphological biochemical and functional characteristics of the quadriceps muscle have been studied in a group of healthy men at different ages Histochemical and biochemical analyses have been performed on biopsy samples as well as determinations of isometric and dynamic strength, contraction velocity and endurance

2 The morphological characteristics of the muscle changed with age (20-65 yrs) in that the proportion as well as the cross-sectional area of the type II fibres decreased, whereas no significant linear change was seen in type I fibre area. Fibre type distribution within the type II fibre population did not change with age

3 Muscle enzyme activity analyses (Mg^{2+} stimulated ATPase MK, PFK HAD CS cytox, LDH H-LDH and M-LDH) revealed that only minor changes occurred in the metabolic profile of the quadriceps muscle in old age That is HAD activity increased slightly with age and M-LDH activity decreased (20-65 yrs) It is suggested that these changes reflect the change in fibre type distribution.

4 Maximum isometric and dynamic strengths in the knee-extensor muscles were *found to increase steeply to the* 20-29-year-old group, remain unchanged to the 40-49-year-old group and then to decline with increasing age (10-70 yrs) A similar age trend was also seen for maximum knee extension velocity (MEV) although the relative changes in MEV were smaller compared to the changes in strength

5 The strength decline seen in old age appeared before any external changes could be measured in muscle volume (thigh circumference) However type II fibre area decreased with age and was also found to correlate significantly with the strength decline indicating the importance of muscle fibre atrophy for the impaired muscular strength in old age. The results from multiple regression analyses suggested however that other mechanisms than the fibre atrophy might be involved in the decline seen in strength.

6 Isometric and dynamic endurance measured in relation to maximum strength tended to increase with age (20-65 yrs) although statistically not significant. These tendencies were in line with and correlated with some of the alterations seen in histochemical and biochemical characteristics of the muscle (per cent type II fibres type II fibre area and M-LDH activity) However maximum strength decreased in old age indicating a reduced isometric and dynamic endurance when measured in absolute terms

7 Patellar reflex measurements revealed an increased total reflex time (TRT) in old age This seemed to be entirely due to an increase in the motor time component i e the time taken for spread of excitation and development of sufficient tension within the muscle to overcome inertia and set the lower leg in motion.

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The Secretory Pattern of Growth Hormone

An Experimental Study in the Rat

By
Staffan Eden

Göteborg 1978

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The Secretory Pattern of Growth Hormone

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The pr sent thesis is bas d on the following pap rs:

I Monoamine gi control of episodi GH secr tion in the r t: Effects of r serpine α-methyl-p-tyrosine p-chlorophenylalanine and haloperidol
S Edén P Bolle and K Modigh: Submitt d to Endocrinology

II Eff cts of apomorphin and lonidine on rat plasma growth hormone aft r pretre tment with reserpine and l ctroconvulsive shocks
S Edén and K Modigh: Brain Re 1977 129 379-384

III Plasma levels of growth hormone in female rat of diffe ent ages
S Edén K Albert son-Wikland and O Isak son: Acta endocr (Kbh) 1978 88 676-690

IV Age and sex relat d diff rences in pisodic growth hormone s cretion in the rat
S Edén: Endo inology 1978 accepted for publication

V *In vitro* ff t of growth hormone on protein synthe is and amino id transport in the r t diaphragm after cut hypophys ctomy
K Albe t so -Wikland S Edén and O Isaksson: Acta physiol scand 1978 I pre

The paper are ref rred to in the text by their Roman ume als

LIST OF ABBREVIATIONS

AIB	α-aminoi butyric acid
α-MT	α-methyl-p-tyro in
CA	catecholamin
CNS	entral n rvous y tem
DA	dopamine
Dopa	dihydroxyphenyl lanine
ECS	l troconvul ive hocks
ECT	l t oconvulsiv the apy
FSH	f lli l timulating hormon
GH	growth hormone
GHB	gamma-hydroxybutyr t
GHIF	growth hormone inhibiting f t (somat st ti)
GHRF	growth hormon r lea i g f t
5-HT	5-hydroxy tryptamine
5-HTP	5-hyd xy tryptophan
ICGH	int g t d con t tion of g owth hormon
LH	lutei izi g hormon
ME	median eminenc
NA	no drenali
NIAMDD	N tional In titute f Arthriti Met bolism and Digestive Di eas
NSD 1015	3-hyd xybenzyl hyd azine
PCPA	p-chlo ophenyl lanine
SEM	tand d rr f the mean
VMN	ventromedi l ucl us

CONTENTS

INTRODUCTION

Regul tion of the se ret ry patt rn f growth hormone (GH)

The proximity f the anterior pituitary to the hypothalamus and the v scul r connections between th two constitut basi f r the hypothalamic control f secretion f hypophysial hormones in luding GH Ind ed hypoth lamic damage pituitary talk se tion or transplantation f the pituitary to th r rgans were rly hown t influence growth (fo ref Reichli 1960; 1966). With the development f ensitive radioimmunoassays f r GH however the mechanisms regul ting GH se retion could be investigated in detail (Uti ger t l 1962; Glick t l 1963) The rly investigations gge t d that GH se retion wa und metaboli control in a fa tors uch as hypogly emia and amino acid i fusion were found to caus an increas in plasma GH level (Roth t al 1963; Knopf t al 1965) GH was therefo asc ibed an impor ta t l in the moment t moment regul tion f carbohydr te and prot in metabolism and feed-back rel tionship between GH se retion and int rmediary metabolism was gge t d (Glick t l 1965) However subsequent studi showed that the pl sma l vel f GH v ri d markedly and pontaneous fl tuations in the concent tion f GH in plasma with no pp rent relationship t physi logical fluctuations in plasma l vel f glucos amin acid o fre f tty cids were bserved In man release of GH was found to be consi tently associ t d with the ons t f leep (Quabbe t al 1966; Takahashi t l 1968) but a v i ty f the timuli uch a trauma r exercise were lso found to cause GH rel ase Moreove secretory episode occurred with no apparent re l tionship to any stimuli (Spit t al 1972) Furthe tudies reve led th t there we spe i diff rence in the regul tion f GH se retion F ct such unspe ifi tre timul te GH secretion in p imats but cause marked de rease i plasma GH level in r ts (Schalch and Reichlin 1968)

Over the last f w yea s Marti and co-workers hav been able t characte ize th se ret ry patt rn of GH in adult "non-stressed r t by btaini g sequential blood sampl from implanted annulae (Tannenbaum and Martin 1976; Willoughby t l 1976; Tannenbaum t l 1976 f ref Martin 1976 Martin t al 1978) Th se tudi s reve l d that GH was se eted int rmittently i pulsatil patt rn i the adult mal r t Peaks i pl sma GH level occurred t regul 3 4 h int rv l and peak level reach d 600-800 g/ml where l vel between peak were below the det tion limit f the assay Moreove

the timing of the peaks with re pect to the light-dark ycl was similar in most animals This ynch onisation in timi g of the secretory episodes of GH was lo t when the animal were housed under conditions with constant light al though th s cretory patt rn in individual animal was still pulsatile (Tannenb um and Ma tin 1976) Subsequent studi s in their l boratory have shown that the cretory patt rn of GH in the ad lt mal rat i independent of physiol gical fluctuations in blood glucose and insulin corticost ron prol tin or sleep-wak rhythms (for ref Martin _t l_ 1978)

The se r tion of anterio pituitary hormones i influenced by hypothalamic factors r l ased into the portal vessels reaching the hormone-producing cells from the blood tream GH secretion is consid red to b r gul ted by *not yet identifi d releasing factor (GHRF) and an inhibiting factor* somatostatin (GHIF) GHIF has been isolated from th hypothalamus and chemically *identified (B aze u _t al_ 1973) and i a pot nt inhibit r of GH secretion* in several speci The evidence for the existence of a GHRF is mainly th t interruption of the hypothalamic-pituitary continuity r lt in a supp ession of GH se retion and not in enhanced se retion as occur with prolactin (f r f Martin 1976)

Th timulating and inhibiting f tor are thought t be released from neurosec t ry neurons in the hypothalamu These n urons are in turn influ ced by synapti input from hypothalami and xtra-hypothalamic regions *(fo ref Reichlin 1974 Martin 1976; Martin _t al_ 1978) In line* with this assumption e th reports of an influen e f monoaminergic neurotransmitt rs on GH se retion Or l administration of 5 hydr xytryptophan (5-HTP) a precurso f 5-hydroxytryptamine (5-HT) ind ce a moderate increase in plasma l vels f GH in man (Imura _t l_ 1973) and monk y (Chambers and Brown 1976) Admini t tion of 5-HTP has also been hown t stimulate GH secretion in the r t (Smythe and Lazarus 1973) Admini tration of L-dihydroxyphenylalanin (L-Dopa) the pre u so of both no adrenaline (NA) and dopamine (DA) induces increases in pl sma GH level in man (Boyd _t l_ 1970) monk y (Jacoby _t l_ 1974) and dog (Lovinga _t l_ 1974) In man apomorphine a DA recept r agonist (Andén _t al_ 1967) i effective in stimul ting GH se retion (Lal *_et l_ 1973) but not i the rhesus monkey (Chamber and B own 1976) or the* dog (Holland _t l_ 1978) Clonidine an α-r cept r timulating gent (Andén _t al_ 1970) timulates GH secretion in man (Lal _t al_ 1975) as well as in other spe i s (Chamb rs and Brown 1976; Ruch _t al_ 1976; Holland _et al_ 1978)

The hypothalamus is rich in DA NA and 5-HT The DA in the hypothalamus is thought t be contained mainly in n uron which rigin te within the *hypothalamus P actically all of the NA in the hypothalamus i contained in* axons and nerve terminal arising f om cell bodies in the brain tem Also 5-HT in th hypothalamus is mainly to be f und in extra-hypothalami n uron originating from the raphe nucl i f the b in stem and the ventromedi l reticul r f rmation f the pons and mesenc phalon (for ref Fuxe and Hökf lt 1969; 1970; B ownstein t al 1976) The localisation and distribution f GHIF in the hypoth lamus re at thi tage well known Cell bodies containing GHIF are mainly p esent in the ant ri r periventri ul r hypothalami area and GHIF nerve t rminal ar found in the median eminence (ME) and the arcuat ventromedial (VMN) and up achiasmatic nu l i (fo ref Hökf lt t al 1978) Indirect evidence based on bioassay le ion experiment r l t ical timulation technique indicates that GHRF i to be found in the VMN rcuat nucl us and the ME (f ref Martin 1976) Each f the GHIF or GHRF containing neuron may be controll d by any f the monoamines as well as of other neurot ansmitters

The important relationship between functions in the CNS and the ndocrine sy tem has been known f a long time Endocrin disorders uch as hypo- and hyperthyroidism re asso i ted with mental symptoms (*Gibson 1962*) The thyroid hormones have been shown t potentiate the the ape ti ff t f antidepre ant drugs indicating an influence f these hormones on monoaminergi neurotransmi ion (Prange t al 1969) Moreove psychoa tive drugs have neuroendocrine ide ff ct F xampl it i well known that p tient tre ted with reserpine chl rpromazine r phenothiazide can develop gal c t rrhea probably due to increased prolactin se retion The ff t f these drug is thought t be due t int rf rence with DA ne rotransmis ion (Wurtman and F rnstrom 1978) *Thus increased knowl dge of the ne ral mechanisms regu*l ting pit itary fun tion has opened the po ibility f an increased und rstanding f changes in the function f the CNS by tudying hormone secretion from the ant rior pituitary

GH growth and intermedi ry metabolism

The impo tanc f the ant ri r pituitary f normal growth was stablished ly i this entury I 1912 Ashoe reported that removal f the ant i pituitary l d to cessation of somati growth Evan and Long (1921) showed that xt acts f bovine pituitary gland produced upran rmal growth

in r ts Smith (1930) developed surgical techniques for hypophysectomy of the r t and also showed that growth in hypophysectomi ed rats could be parti lly re tor d by hypophyseal transplants A highly purified protein from bovine pituitari s that produced somatic growth when administer d to hyp physecto mized or normal r ts was is lated and characterized in the 1940 (Li t l 1945; Wilhelmi et l 1948) Thi protein h d little or no ffect on adrenals thyroid or g nads and was called growth hormone (GH) or somatot pin

Early studi s indicated that the anterior pituitary als was important in the regulation of int rmediary metabolism The diabetic st t f pancreat- ectomi d dogs wa improved aft hypophysectomy (Houssay et al 1931) and xtra ts of the anteri pituitary was shown to cause hyperglycemi when in j cted to intact animal (Young 1937) Burn and Ling (1929) showed that pitui t ry xtra ts increased ketonu i in rat Teel and Cushing (1930) ob erved *a de rease in urinary xtraction of nit og n and ph sph rous* and a decrease in the blood concentration of nitrogen after administration f pit itary ex- tracts to rats Subsequent studie with purifi d GH bowed that GH influenced carbohyd te protein and lipid metabolism Thus prolonged treatment with GH was found to be s ociat d with hype glycemi in rmased catabolism of f t as well s protei anabolism and nhan d growth (f r ref Ko tyo and N tting 1974 Goodman and Schwart 1974; Alts uler 1974)

There i however no simple rel tionship between GH secr tion, growth, and i t rmedi ry metabolism Fo example levels of GH in plasma d ri g late f tal lif and in the newborn are high (Kaplan t l 1976) but absence f GH p oduces littl hange in fetal growth (fo ref Che k and Hill 1974) GH le vels then de line d ring the neonatal pe iod but GH deficiency during the first ye f lif i associat d with growth retardation (Br sel et l 1965; Rimoi t al 1968)

The ltimate growth re ponse t GH of ta get tissue consists f an in- re i the ynth f prot in and ucl i cids (f r ref Ko tyo and Nutti g 1974) The timulati g ffect of GH on protein synth is i ti ue from hypophyse tomi d animal is r pid in onset and det ctabl within 20 min aft the admini tr tion f the hormone The stimulation of ucl i acid syn the i by GH i however not observed until se r l hours aft GH admi i t tion Thus GH i it lly stimul t s prot i ynthesi by i e i g the activity of ribosome alre dy p sent and consequence independently of this ffect of GH ll p t in ynthet i units ar activ t d (f r ref

Kostyo and N tti g 1974) The stimulatory eff ct of si gl injection of GH on prot in synthesi has b en shown to last f 24 h (Kosty and N tti g 1973) The appare t long d ration f the eff ts of GH on protein ynthesi indicat that rapid change in the plasma concentration of the hormon might b f minor importa ce f r the anabolic eff ts f GH

Mo t tudi s on the ffects f GH on intermediary metabolism have however been pe formed in animals hypophysectomi d several day prior to tudy and usi g rel tively larg doses or high concentrations f th hormon The ff ct f GH regist red under ch ci umstances might not be representa tive f th physi logical actions f the hormone in the intact animal The ffect f a rge f GH in pl sma on tis ue in the intact organism may th re fore be diff re t from those regi t ed aft r dmi istratio f GH t hypophy ectomized animal An in rea ed understandi g of the reg latory mechani m f GH secretion and f normal variation in th s retion of GH may be lu able f r the lucidation f the mechanism f ti f the hormone

AIMS OF THE PRESENT STUDY

The high concentration of monoamines in the hypothalamus and the ME and the influence of monoamine on GH secretion indicate that monoamines are of considerable importance in th regulation of GH secr tion The role of monoaminergi neuron in the regulation of the secretory patt rn f GH is however not l ar The present investigation was initiated to evaluate the role f monoaminergi neurotransmi ion in the control of pisodic GH secretion in the t (Paper I)

It has be n shown that th behaviour l re ponse to CA goniste in mi e is enhanced afte pretreatment with electroconvulsive shocks (ECS) (Modigh 1975) T valuat if studies on GH secretion *could be used a a tool to study* v ri tions in sensitivity of monoaminergi r cept r the ffects f pretreat ment with ECS on CA induced GH rel ase were tudied (Paper II)

In a recent report from our l borat ry it wa hown that GH stimulat d amino acid transport and prot in synthesis in di phr gms of young female r ts (10 20 day old) whereas these ff ts wer poor r inconsi t t in younger (10 day) and older (> 20 days) animals (Albertsson-Wikland and Isaksson 1976) It ha been shown that plasma GH l vel in the rat hange d ring the fir t weeks f lif (Rieutort 1974; Stros r and Mialhe 1975) The present experiment were initiated to evaluat if the vari tions in tis ue sensitivity to exogenous GH with age were associated with v ri tions in the se retion of the hormone (P per III) Sinc it has be n hown that the ecretory patt rn of GH is different in adult female r ts ompared to ad lt mal (S unders t l 1976) the devel pment l change i the s ret ry pattern of GH were studied separat ly in young femal and mal ats (Pape IV)

The poor incon ist nt ff t f exogenou GH on ti ues from normal animals have been ribed t th long-la ting ff ts of endogenous GH (Kostyo and K tting 1974) Recent expe iment in o r l bo t ry have indicated that the d r tion f th ff ts of GH on amino id transport and prot i synthe is in skel tal muscl f young (18 day-old) rats i hort r than those in ats that have be n hypophy ectomi d several days pri t study (Albertsson-Wikland and Isaksson 1978) In the present xperiment the ff t of exogenous GH on amino acid t ansport and prot in synth i I the diaphr gm mus le we studied t sho t time int rv l ft r hypophy tomy t ev luat th time--co rse f the developme t f re pon iveness f the tis ue to GH ft limination of nd genous GH (P pe V)

The purpose f the present tudy can thus be ummarized f llows:

1 to study the rol f monoaminergi ne rotransmission in the control f normal GH se retion

2 t tudy if pretreatment with ECS wa associated with ch ge in the CA induced rel ase f GH

3 t cha ct rize the se ret ry pattern f GH in f mal and mal rats of diff rent ge

4 t tudy the time-cou se f the devel pme t f ti ue respon ive- ne to GH fter cute hypophysec tomy

METHODOLOGICAL CONSIDERATIONS

It was early recognized that singl measurements f GH in plasma were f little value f r diagnostic purposes Therefore, various stimulation t sts h ve been used, for xampl in th diagnosi f pituitary in fficiency (Frasier t al 1974) These te ts may however mo e reflect the secretory e serve of the pituitary than the "physiol gical GH secretion (Reichli 1974) It i now known that GH se retion is episodic rather than ontinuous and that the half lif *of GH in plasma both in man (Gli k t al 1964) and in the rat* (Frohman and Bernardis 1970) is relatively sho t This in turn nec sitates repeat d measurements of GH in plasma from the ame individual to clarify normal variations in GH secretion Moreover the secretion of GH i labile *fun tion influenc d by various stimuli which can ithe enhance o i hibit GH* se reti n (f r f INTRODUCTION) These fi ding mak dequat ontrol on ditions impe ative in studi s on GH se retion and GH gul tion

Experime tal animals

Sp gue-Dawley rat purchased from Anti imex St ckh lm were u d in all xpe ime t Th imals were delivered t least 3 day bef re they were exposed to any treatme t or used i experiments They we housed under con trolled onditions in a room with rtifi ial light betwe 0500 and 1900 h Food (Type R3 Anti imex Stockholm) and t p water were freely available in mo t expe iment Animals younger than 20 day were ompanied by a mother (P pe s III and IV) Eight pups f the same se were d live d with mother I ll xpe iment body weight wa reco ded daily nd r t with a normal growth t wer on idered he lthy

Blood sampli g procedure

Singl blood ampl s Sin tress i known t inhibit the rel se f GH i the at (S hal h and Rei hli 1966; 1968; Dun et l 1973/74; T ry t l 1976) g at ca e w tak t minimi the i fluence f tr asso iated with blood ampli g All forms f anas the ia d ing blood ampli g were omitt d al diff t f rms f anae the ia have bee shown t i flue GH se r tion (Schal h nd Rei hli 1966; Tak ha hi t l 1971) lso i young animal (Str se nd Hiahl 1975) I the pr se t xperiment the animal were daily weighed handl d nd remov d f om thei g f hort pe iod f time

When single blood sample were taken the r t were gently removed from thei cages and blood wa btained either from the trunk aft rapid de api tation r from the tip f the tail aft r a quick cut with a knife Plasma level f GH were i th same range irrespective f the technique used fo btaining singl blood sampl s (cf Tabl 1 Pape III) GH levels in rat sampl d from each cage may however have been influenced by tress induced when handling the cages r picking the animal out of their cag s Howeve GH level in th r ts sampled sequentially from the same age were not suc ce ively lowered indicating that the sampling technique pe se did not i fluence the pl sma conce t ation f GH By btaining blood ampl from th tip of the tail plasma GH levels could be measured in the same animal on different oc asions In the present tudy blood sampl s were btained at 3 4 day interval in rd r to ns re th t previous sampling did n t aff t GH level

Sequential blood sampl s from implanted annulae In younge animals (≤ 30 days ld) the cannulae were implant d via the right ext rnal jugular vein into the superior v cava (Paper IV) In lde animal intra-aorti cannul were implanted a described by Popovi and Popovic (1960) In the auth r s hands arte ial cannulae were easi to keep pen and could be us d f r repeated sampling f blood on consecutive days and wer therefore used when pos ibl On each day afte the ope ation a p lyethylene tube (PE 50 void volume 0 1 ml) was conne t d t the cannul and brought out through the top f the age This tube wa subsequently used to take th blood sampl s The animal were handl d daily on sever l occa ion to dapt them to th sampling p ocedure In preliminary inve tig tion (P pe II) sequenti l blood samples were taken on the day after the operation Methodol gical xpe iment revealed howeve that th se retory pattern f GH in these animal wa different compa ed to animals dapted fo seve l day to the sampli g proc d s In Fig 1 the re ults from on methodological expe iment are hown I this experime t blood sampl were obtained from dult male r t (90 day old) at 30 mi intervals fo 3 - 4 h periods on 4 consecutive days aft the implantation f the cann la The day fte the operation peak level of GH were markedly reduced On the second thi d and fourth postope ative day pe k level of GH we highe and plasma level were low between peaks The apparently normal se retory profil s occurred con omitantly with gain in weight All animal included i ubsequent xperiment were allowed to recove fo t

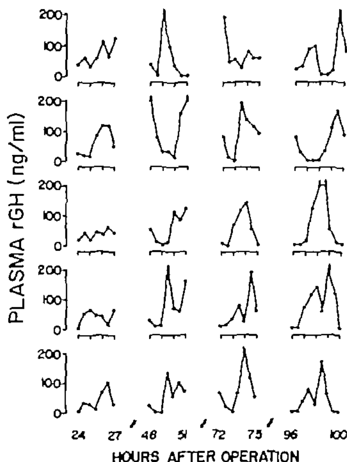


Fig 1 Effect of surgical trauma on the secretory pattern of GH in adult mal rat Blood sample were obtained at 30 min interval for 3-4 h fo 4 consecutive days following surgery Arrows indicate level > 200 ng/ml

l st 4 days bef re experiment Animals with the following criteria were considered healthy":

1. Gain i weight from the second postoperative day
2. Body weight on the day of blood sampling equal to or over preoperative body weight
3. *Gain in weight or stable body weight on the day after blood sampling*
4. Normal behavi r as assessed by gro s observation and lack of signs of stre uch as piloere tion r periopheral vasoconstriction d ring blood sampling

The third and fourth criteria were not consid red in the studie on monoami nergi control of GH se retion (Paper I) if drug were used that affected normal behaviour body weight gain in animals not exposed t blood sampling The importance of the fourth crite ion i illust ted in Fig 2 In this experiment 45 d y-old femal r ts were accidently exposed t blood lo because f a leakage in the conne ting tube The blood lo s was associat d with piloraction and peripheral vasocon triction and lso with marked inhibition f GH secretion

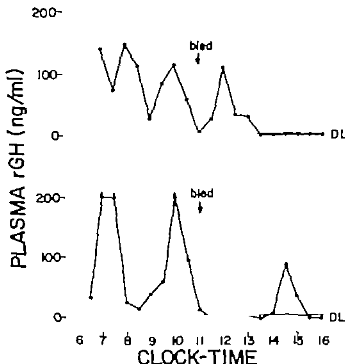


Fig 2 Effect f bleeding on GH secretory profile in 45 day-old female rats. Blood samples were obtained from 2 rats at 30 min intervals. At 11 00 both rats were exposed to blood loss (bled) By gross observation, the bleeding was followed by peripheral vasoconstriction and piloerection and GH levels in plasma were depressed concomitantly Arrows indicate levels 200 ng/ml. D.L. represents the detection limi f the assay (2 SD above zero dose response in the standard curve)

Det rminati n of plasma lev ls of GH

The devel pment of r dioimmunoassay methods with sensitiviti s th t by far exc ed that of bioassays ha enabl d detailed studi s of variations in the l vels of diff rent hormones in pl sma Se sitive adi immunoassays for rat GH have been available for more than 10 ye r (Park r et al 1965; Schalch and Reichlin 1966) Determination of plasma levels of GH by radioimmunoassay probably refl cts the amount f GH released from the pituitary inc the half life of the hormone in pl sma i only 5 - 20 min It i howe er not cl r if GH in pl sma measured by radi immunoassay i the phy iologically ctive entity (for ref Kostyo and Isaksson 1977) GH ppears in differe t immun logically activ forms in pl sma (Goodman t l 1974) but the biologi cal activity of thes diffe ent forms i not known Moreover there re i di cation of discrepancy between plasma levels of GH dete ted by radi immuno-assay compar d to that measur d by bioas y (Elli and Gri deland 1974; Ellis t l 1976) Othe i vestigat rs have however reported good orrel tion between GH l vels me s red with bi say and radi immuno say (for ref Kostyo and Isaksson 1977) and further studi s re required to resolve th e i ues

In the present xperiments pl sma l vel f GH were d termined i g a doubl antibody t ch ique devel ped in coll boration with the Departme t of Physi logy Emory University Atlant Georgi The a say wa devel ped t ure high sen itivity thereby allowing ac urat timation f plasma GH in small sampl volumes Highly pu ifi d rat GH (R 1051 A) with a ported bi logi al a tivity f 2 IU/mg as assessed by the body weight gai y was used as r fe enc p par tion in the stand d curve f th y Thi highly pu ifi d prepa tion wa g nerou gift from Dr A E Wilhelmi The same hormone wa iodinat d with I^{125} using the la toper idase method (Thorell and Johan o 1971) About 20 g f the h rmone w s iodinat d very 3 months Immedi t ly ft iodi tion the radioa tive mat ri l w s plac d on Sephadex lumn (600 9 mm i d) el t d with 1 % bovine serum lbumi in 0 05 M phosphate buff r (pH 7 5) One ml fraction wer lle ted The r dio-acti ity in each fraction was mea ured and thre peak were obtained (Fig 3) Ea h fraction was t t d fo binding to antibodi s t r t GH Maximal binding was found i the se ond peak Th s fra tion wer k pt od t r d f o t -30°C i sub ampl s The fir t peak wa con idered t be aggrega-t s of GH and the third pe k the free iodine P i r t ach radi immuno-a s y the labell d hormon w r purified on the same column (Fig 3)

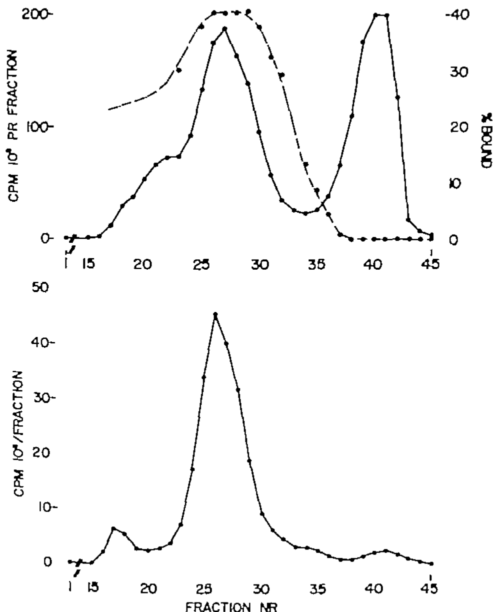


Fig. 1. Gel filtration of labelled rat GH (1051 A) Afte iodination the hormone was peri fied (upper panel) on Sephadex Column (0 75 600 9 mm i d) luted with 1% bovine serum albumine in phosphate buffer (0 05 M, pH 7 5) One ml f actions wer collected and the radio- activi y in each fraction counted (•—•) The binding of each fraction (•--•) was sted by incubating ~ 10000 cpm with A-rGH 8-3 (1 200000) for 24 h The binding was 40 % in frac- tions 26-29 These fractions were pooled and stored in subsamples at 30°C for subsequent use A subsample of the fractions pool d and collected after the f rst purifica ion was repurified on the same column (lower panel) before ach assay One ml fractions were collected and the adioactivity in each f action counted Fractions with the highes adioactivity were pooled and used in the radioimmunoassay for ra GH.

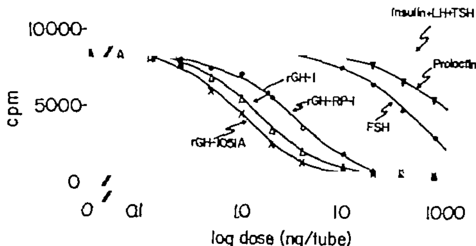


Fig 4 Specificity of rat GH radioimmunoassay Labelled rat GH was incubated with A-rGH S-3 (1 100000) and with different concentrations f the f llowing hormone at GH 1051 A (Wilhelmi) at GH-I (NIAMDD) at GH RP-1 (NIAMDD) at FSH RP-1 (NIAMDD) at prolactin RP-1 (NIAMDD) at LH RP-1 (NIAMDD) bovine TSH (NIAMDD) pork insulin (MC-S-821506; Novo Co- Copenhagen)

Specific antibodi s against rat GH (Anti-Rat GH-S 3) gene ously uppli d by the National In titut of A thriti Metabolism and Digestive Diseases (NIAMDD) were used at final dil tion of 1:1000 000 - 1 200 000 givi g abo t 40 % bi ding when incubated with the labelled hormone f r 24 h at 4°C T t t the spe ificity of the antibodi the binding of the labell d rat GH was tested in the presenc of othe polypeptide and pit itary hormone p parations (Fig 4) As shown in Fig 4 the R 1051 A r t GH prepa tion was highly potent and 2 3 time more so than the other rat GH preparation (NIAMDD GH I and RP I) Rat prola ti (NIAMDD RP I) and r t FSH (NIAMDD RP I) interf r d slightly at very high concentr tions with the bindi g f the labell d hormon t th antibodi The the hormone preparation (NIAMDD t LH RP-I and bovine TSH B 2) and pork ins lin (MC S-821506 Novo Co Copenhagen) did not bind to the anti bod es

Pre ipitati g ant bodi s against normal monkey serum were raised in rabbits a co di g to V ituk itis et al (1971) Boost r vaccinations we e gi en t 3-4 month interv l and the animals were bled at 3 4 week i t rval After each bl edi g the bi ding p ity of the separat d serum was t sted The serum wa used at a dil tion of 1:7 1:15 Repeat d thawi g of the serum induced a rapid l s f binding pacity and wa av ided

In the a s y the tandard curve consist d of tub with 0 0625 - 4 0 ng/tube Plasma level of GH were determined in duplicat r triplicate samples of 10 µl plasma In some xperiments (paper IV) 5 µl of the plasma sampl were diluted with 5 µl of plasma from hypophysectomized rat and tored frozen Ten microliters of pl sma from hypophysectomized r t were added to the tubes in the standard curve A 1 git v log plot was used f r the calculations (Fig 5) and this plot was line between 0 05 - 2 0 ng/ tube in all says The det ction limit of the assay (2 S D above ero dose in the tandard curve) was lway < 0 05 ng/tube Whe available (paper IV) plasma ampl s with a concentration of GH ver 200 ng/ml were diluted and reas ayed and re therwise expressed >200 ng/ml In all assays the slope f the graphs f the tand rd curve were cal ul ted and half maximal bi ding (B/Bo 0 5) was 0 45-0 50 ng/tube and B/Bo = 0 9 was 0 05-0 10 ng/tube

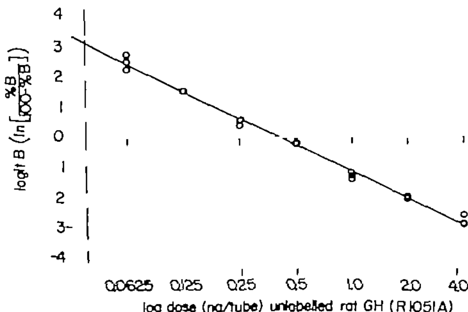


Fig. 5 Standard curve from rat GH radioimmunoassay expressed in logit-log coordinate. Labelled rat GH was incubated with monkey antibodies to rat GH monkey(A-rGH S-1, final dilution 1 200000) with diff rent concentrations f unlabelled hormone for 24 h at 4°C. Hatched vertical line represents the detection limit (2 SD above zero in the standard curve 0.023 ng/tube)

Tabl 1 Plasma level of GH measured in diff rent dilutions

Amount of plasma in assay (µl)	Mean plasma GH concentration (g/ml)	% of level measured in 10 µl
5	46.5 12 2	51 8 2 5
10	45 3 10 3	—
20	45 7 10 4	205 1 4 1

Blood was obtained from 20 rats by rapid decapitation. Plasma was separated and GH levels were measured in duplicat f 5 10 and 20 µl of plasma. Equivalent amount of plasma from hypophysectomized rats were added to corresponding tubes in the three differen tandard curves

The within-assay variability f r t iplicate and dupli t det rminations was 5 - 12 % (coefficient of v riation) The betwe n-as y variability was e timated by including triplic te sampl of 3 4 plasma pool in each as y The coefficient of variation f r the between y variability wa 10 - 26 % the highest variation in the lowe range f the assay

Since GH is known t appear in diff rent immun logi al forms in plasma 5 10 and 20 µl sampl s of th ame plasma were as ayed in some expe iments (Tabl 1) The dilution slope v s p all l to that of the standard c rve ugg ting that no gr ly different f rms f t GH ppear d in the plasma Diff rent amount of unlabelled hormone were l o added to tubes containing ithe 10 µl of plasma from hypophyse tomi ed rat 10 µl f pool d plasma from normal ts (Tabl 2) The re v ry f the ass y w approximately 100 % (ang 88 - 112 %)

Table 2 Recovery ate in rat GH adi immunoassay

Added amount GH (ng/tube)	Tube with hypox plasma: Amount of GH measured (ng/tube)	Tube with hypox plasma: Recovery %	Tube with 0.45 ng GH/tube: Amount f GH measured (ng/tube)	Tube with 0.45 ng GH/tube: Recovery %
0.25	0.28 0 01	112 2	0 70 0 03	100 12
0 05	0.48 0 01	95 1	0.89 0 03	88 6
1 0	0 98 0 03	98 3	1 49 0 04	104 3
2 0	2 10 0 08	105 4	2 49 0 05	102 3

Diff rent amounts of GH (R 1051A) were added to iplica sampl with plasma from hypophyse tomized rat and to plasma with 0 45 ng GH/ ube as determined by the at GH adioimmunoassay The amoun f GH was in each ype subsequently determined in the say

Drug administ tion

All drug were given in neutral s lutions by the intraperitone l route in volumes of 10 r 20 ml/kg Control animals received 0 9 % alin The following drugs wer used:

Reserpine (Serpasil; CIBA Stockholm)
Apomorphine HCl x 1/2 H_2O (Sand Basel)
Clonidine HCl (Boehringe Ingelheim Stockholm)
α-Methyl-p-Tyro ine methyl st (α-MT (H44/68); HH sl Göteborg)
p-Chl rophenylalanine methyl ster HCl (PCPA (H69/17) Hässl Göteborg
Haloperidol (Hald l; Leo Hel ingborg)
3-Hydroxybenzyl hyd azin HCl (NSD 1015; Smith & N phew Re arch Ltd London)

El troconvul ive hocks (ECS)

Group f animal were pretre ted with on ECS daily f 7 day de ribed in paper II

Motor Activity

In some xperime t (p pe I) the ff t f diff rent drug on behaviour was stimat d by measuring mot tivity by means f "M/P 40 F El ctronic Motility Mete (Mot on Produ t Stockholm) These re based on 40 condu tive sensors rranged in 5 ows f 8 cell with a cente to center di tance f 40 mm The cell are covered by translucent pl tic box. The light source i regular incand scent lamp mount d 115 cm over the photoconductive cell The recording were ta ted 30 sec after inse ting the animal in the t t cage and moto tivity was recorded during 30 60 min and regi tered as ount per 10 min The measurements were per- f rmed between 1000 1400 h

Model used t timate monoamine synthe i

CA and 5 HT ynthe i we det rmined by measuring 3 4-dihydroxyphenyl- lanine (Dopa) and 5-hydroxytryptophan (5-HTP) accumulation respe tively 30 min ft the admini t tion f NSD 1015 as previously de cribed (Carl son t l 1972)

Determination of br in conc nt tion of monoamines and monoamine precursors

B in l vels of monoamines and their precurs rs were measured fter purification accordi g to Atack and Magnusson (1970) by the following spectrophotofluorimetri analyses: DA (Atack 1973) NA (Bertler t l, 1958) 5-HT and 5-HTP (Atack and Lindquist 1973) and Dopa (Kehr t l 1972)

Acute hypophyse tomy

Female ats 30 days ld were hypophysectomized under ther anaesthesi by the parapharyng al approach (Smith 1930) This t chnique has been used fo many y ars in this laboratory (Ah én and Hjalma sson 1968) The complet nes of the hypophysectomy was test d by measuri g the plasma l els of GH t various time int rv l after the oper tion There were no detect-abl l vels of GH in pl sma t 2 6 and 24 h aft r the ope tion (pape V) In orde to evaluat if GH l aked out i to the blood tr am d ring the operation plasma GH was measu ed in separat experiment at 0 10 20 30 60 and 90 min after the removal f the gland Ther were no det t bl l vel of GH i pl sma f any f the r ts (5 in ach group) which clea ly shows that no such l akage occurred

To bt i appropriat control t the hypophysectomiz d r t groups f animals were sham-operated The ope tion was identic l to that perf rmed when t were hypophyse tomized exc pt that the pit itary gland was not removed du i g the urgi l procedure

Incub tion pro d re and det rmination f amino acid t ansport and in corpo tion i t p t in

In pape V the ffect of GH i vitro on amino acid transport and prot i ynthe i i the at di ph agm wer tudi d aft r acut hypophysectomy These two pa amet rs have been widely used in studies on the eff ts of GH and were theref re chosen to implify comp isons with pre ious re ults Femal rat (ontrol sham-operat d and hypophysectomized) were kill d by rapid decapit tion and blood w collected from the trunk fo subseque t det rmination of pl sma GH Intact hemidiaphr gms were prepared ac ordi g to Ko tyo and Knobil (1959) The paired diaphragms were washed for 10 min and then tran f rred to othe fl k with r without

bovine GH (NIAMDD bovine GH-18) at a concentr tion of 5 µg/ml They were incubated fo additional 30 min and then processed to me sure the di t i-bution rati of the non-utilizable model amino acid α-aminoisobutyric acid (AIB) and the incorpo tion f labell d phenylalanin into diaph agmal protein The methods used have been described by Hj lmarson and Ahrén (1967) and Alba tsson-Wikland and Isaksson (1976)

Stati tical procedur s

Values are given s means ± SEM Significances between mean were calcul ted by Student t-t t (Snedeco 1956) o whe more than two groups were comp red with analy is f variance f llowed by Student-Newman-Kuehl s multipl range t t (Woolf 1968) P-value l s than 0 05 were considered significant

Since normal di tribution f the group and homogenou v rianc are required f the use f these t ts the f llowing methods we also used to check significance of diff rence in GH plasma level between different group

Wilcoxon t t (Colquhon 1971) was used when two group were ompared

Fishe permutation te t (Fisher 1960) was used t v luate diff rences between group on ch sampling ca ion Those ignificance of analogous comparison were pooled to btain compound ignificance (Odén and Wedel 1975)

Transf rmation of aw GH data to l g ithms was used t obt in homogenous variances (Woolf 1968) -Conventional parametri t ts were then used f omparisons between mean

Since these meth d gave re lt identical to those obtained with raw GH data and conventional parametri te ts these alculations were sometime omitted (Papers I and IV)

RESULTS AND CONCLUSIONS

Monoaminergic control f pisodic GH se retion (papers I and II)

In these experiments the effects on GH secretion f pharmacological agents which are known to interf re with monoaminergi n urotransmission were st di d Adult mal rats (3 months old) were used to simplify comparisons with results obtained in ther labor tories (for ref Martin et l 1978) Sequenti l blood sampl s were btained for periods of at l ast 3 h so that major peaks in the plasma concentraion f GH could be det cted

Admi istration of a high dose of reserpine (10 mg/kg) was found t compl tely inhibit GH secretion for at least 15 h and fter 24 h GH levels we e markedly depressed At 36 h after the inj ction apparently normal episodic GH secretion had reappeared (Fig 6) Admini tration f reserpine induced within few hours pronounced psychomot r inhibition hunched back position and blepharospasm At 24 h after injection of eserpine there was marked reduction of brai levels of CA and 5 HT At 36 h aft r injection the behavi ural inhibition was less complete whereas brai l vel of CA and 5-HT still were markedly depressed The complete inhibition of GH se retion could be prolong d by repe t d administration f reserpine a r gimen that lso po tponed the reappear anc of peaks in the plasma onc ntration of the hormone (cf Fig 3 Paper I) and correspondingly prolonged th har ct ris ti behavio al eff cts f the drug The time chedule for the inhibition and recovery of GH secretion fter reserpine is i good ag eement with the ff ts f the drug on cent al and periphe al mon aminergi n urotransmission

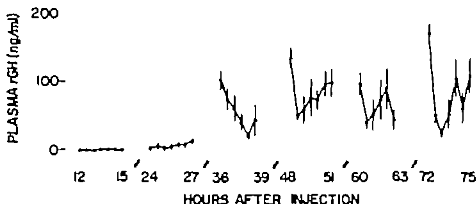


Fig 6 Effects of ingle injec ion f serpine (10 mg/kg i p) on plasma GH Blood ampl were obtained 30 min int rval f 3 h at 12 h interval for 3 consecutive days Each point represents the mean of 12 observation Vertical bar represent SEM

(for ref Ca lsson 1965)

Since reserpine inhibits neur t ansmission in all types f mono-aminergi neurons specific synthesis inhibitors were used t valuate the rol of each f the monoamines in the control of GH cretion Admini stration of α-MT a tyrosine hydroxylase inhibito partly inhibited GH secretion which is in accordance with previous bservations (Moell r t 1 1976 Du and at al 1977) Afte α-MT moto ctivity was partly inhibit d and br in levels f CA as well s CA synthe is refl t d as NSD 1015 in duced accumul tion f Dopa wer depres d B ain l vels of 5-HT and 5-HT synthe i reflected as NSD 1015 induced ccumul tion of 5 HTP were un-aff t d after treatment with α-MT

An impo tant rol f CA ne rotransmi ion in the ontrol f pisodi GH se retion was furthe sub tantiated by the finding th t animals given halope idol DA and NA receptor antagoni t had very low level of GH in plasma with a total abse ce f epi odi secretion f the hormone I line with these result are the findings that phenoxybenzamine an α recep-to blocking *agent cause compl te suppre ion of epi di GH secretion* in the t (Martin t 1 1978 a) and that the DA receptor antagoni ts ( ) butaclamol (Willoughby t al 1977) and pimo ide (Martin t 1 1978) have inhibit ry ff ts on pisodi GH rel ase The ff t of the DA antagoni t was howeve 1 pronounced than that f phen xybenzamin (Ma tin t 1 1978) indicating that α-recepto mechanisms re more impo tant that DA receptors in the control f pisodi GH se retion in the t Thi oncept is furthe trengthen d by the findi g i the p se t tudy that lonidine induced GH rel ase in reserpine pretre ted animal whereas apomorphine wa ineff ti in dose that duced p ychomoto timul tion and tereotypie Furthe previous tudi have hown th t tre tment with lonidin re tor episodi GH se retion in t pretre ted with α-MT whereas pomorphin is ineff tive (Durand t al 1977)

Admi i t ation of PCPA an inhibit f tryptophan hydroxyl se t repeated interval bef re experime t caused marked reduction f b in l vel f 5-HT and an inhibition f 5 HT ynthe i B i l vel of CA and CA synthe i were only lightly reduced aft PCPA Admi i tr tion f PCPA had howeve no ff t on pisodi GH s retion Tre tme t with PCPA has previously been reported t inhibit GH se tion i the t (Marti t 1 1978) These i ve tig t rs found th t 14 19 h aft the last inj tion

of PCPA GH peaks were reduced comp red to controls In the present study the la t inj ctions of PCPA were given 24 - 27 h before the experiments The di crepancy between the prese t results and those of Martin and co--workers might be explained by the fact that PCPA besid s its ffect on 5-HT ynthe is induce a short-lasting inhibition of CA synthesis (Koe and Wei smam 1966; McGe r t al 1968)

The compl te inhibition of GH secretion after treatment with reserpine thus seems to be d rived from the effects of the drug on CA n urons rath r than 5-HT neurons Moreover clonidine but not pomorphine could reverse the reserpine induced inhibition of GH secretion indicating that activation f α-adrene gic receptors is important and perhaps also nec ssary for episodic GH secretion to occur in this speci s

Eff ts f ECS on CA induced GH cretion (Paper II)

Pretreatme t with ECS x VII had no apparent effects on GH se retion in therwise untr ated animals In this prelimi ary inve tig tion sequenti l blood samples were bt ined the day after implantation of the cannula The secretory profil in these animals were diff rent from those in animals allowed long r re overy pe iod in that peaks occurred at irregular inter-val and peak l vel were lowe Subsequent studies perf rmed t l ter time int rvals afte pe tion have not reveal d a y significant ffects of ECS on the se r tory pattern of GH (Boll t l 1978)

Admini tration f reserpine inhibit d GH se retion compl t ly in ECS pretreated animal s in control Th combined tre tment with pomorphine and clonidine given 4 h fter reserpine inje tion induced a significant release of GH in all animal pret at d with ECS x VII and in 7 of 10 animals p treat d with ECS the peak plasma GH levels were over 100 ng/ml In control animal the GH rel se after administr tion of the combined tre tment with lonidine and apomorphine wa l s pronounced compared t that i ECS pr tre t d animal and peak plasma l vels were over 100 g/ml in only 2 t of 7 animals Thus the GH rel se induced by the ombined treatme t with pomorphine and lonidine w mark dly nhan ed by pretreat ment with ECS indic ting an i re sed se sitivity of CA receptor involved in the regul tion f GH se tion after ECS

The secretory patt rn f GH in female and male ats of diffe ent ges

In all age group studi d plasma levels of GH vari d markedly (cf Fig 2 Paper III and Fig 1 Paper IV) There was however a marked variation in plasma GH levels with ge in both femal and male rats (Papers III and IV) In 10 15 day-old rat l vels anged from < 5 ng/ml - 150 ng/ml The levels f GH in plasma declined and ranged from < 5 - 75 g/ml in 20 - 22 day-old animals Level we gain higher in rats 30 days old and older and ranged from < 5 ng/ml - > 200 g/ml These bserv tions are in good gr ement with previous report that there is pid rise in plasma GH in rats from the 18th day f gestation to high l vels at birth and that GH lev l then r pidly decrease reaching the lowe t l vel at 15 - 20 day of age During the prepubertal and pubertal periods (25 - 50 days f g) l els gain incr as (Dickerman t al 1972; Ei utort 1974; Stro ser and Miahl 1975; Oj da and Jameson 1977)

There was no diff rence in the increase in body weight betwee femal s and males up to day 20 Thereafte the inc ease in body weight w higher in males Thi diff rence in body weight gain developed concomitantly with a difference i the plasma levels of GH In mal s an incre se i plasma GH level was observed between day 20 and 25 whe as the plasma GH levels in femal increased between day 25 30 (Fig 7) Also after day 30 the increase in body weight was more pronounced i mal s compar d t femal There was howeve no diff ence in the ang and di t ibution f plasma GH level between male and femal ged 30 90 d y

In de to tudy the se retory patt rn of GH i femal and male at during development sequential blood samples were taken from individual ats with implanted cann lae The results from these tudi s learly showed that GH was secreted epi odically in pr pube tal r t Ther were howev ignificant diff rence in the se retory p ttern f GH between femal and mal r ts and between ats f diff re t ges

In 22 day-old ts GH was secreted in g l episodes t 3 4 h i terv l but peak l vel were lowe comp red to lder animal and did not xceed 70 ng/ml There was no pparent diff rence in the se retory pattern of GH between femal and mal t thi ge

In lde mal s (30 45 and 90 day ld) GH wa e ted pi odi ally and peaks in the pl sma concentration of GH re ched 200 - 300 ng/ml P ks occurred t regul 3 - 4 h i t rv ls Between peak level f GH wer l w

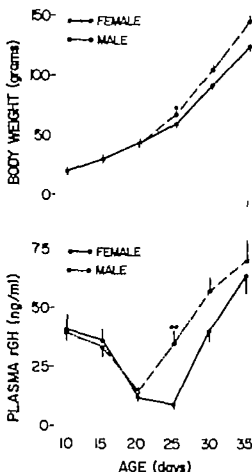


Fig. 7 Body weight (uppe panel) and plasma GH level (lowe panel) in female and mal rats f different age The ats were bled from the tip of the tail tarting on day 10 Eigh female and 8 mal ats in 2 litt rs were bled at 08 00 the next 2 litters at 09 00 the next 2 at 10 00 te. The same schedule for blood mpling was used when the at were 15 20 25 30 and 35 days 1d A t tal of 128 ats (64 femal and 64 mal) were included Each poin represents the mean of 64 observations Vertical lines represent SEM.

p 0.05 vs female of corresponding age

** p 0 01 vs 25-day-old female

(5 g/ml) P aks i pl sma GH l vel wer seen t the same time in mo t mal s indicating a timi g f the se retory episodes of GH between animals The sec et ry p ttern of GH i mal rats 30 d y and lder s en in the present tudy i thus very imil t that b rved in ad lt male rats by Martin and o-workers (f r r f Martin 1976)

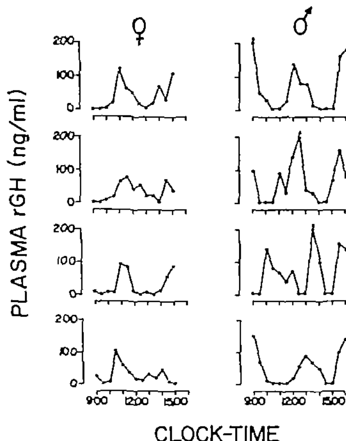


Fig. 6. Individual GH secretory patterns of 4 femal (lef) and 4 mal (right) rats 30 d ys ld. Blood samples were obtained at 30 min intervals fo 6-7 h All animals were sampl d over the same clock-time interval Arrows indicate values 200 ng/ml

In femal (≥ 30 d y) the se ret ry p tt rn f GH was diff r nt compared to mal f corre pondi g ge In 30 day-old t GH w s released episodi lly t ppro imat ly 3 h int rval in both femal nd males. Peak level f GH were howeve ignificantly highe in mal compared to femal (Fig 6) In 45 day -old femal there were pl ual peaks in plasma GH wi h high con ent ation f GH (200 300 ng/ml) which occurred t shorte i rv ls compared to mal s and the v ppare timing f he peaks between animal (f Fig 6 and 7 ... and 7

Paper IV) In 90 day-old femal rats episodic peaks in the plasma concentration of GH occurred at irregul r frequent intervals and peak l vels were ften lower compared to 90 day -old males Between peaks GH levels were often higher in 90 d y-old female compared to males (Fig 9) A differen ce between the secretory pattern of adult female r ts compared t ad lt mal has previou ly be n report d (Saunders et al 1976)

The results from the present study demon trat that GH is secr ted pisodi ally in prepubertal as well as in pube tal and adult r ts For

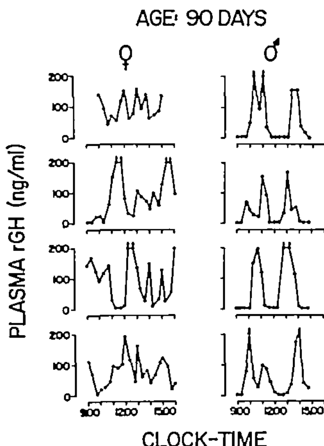


Fig 9 Indi idual GH secretory patterns of 4 femal (l f) and 4 mal (ight) ts 90 days ld. Blood ampl were obtained at 20 min intervals for 6-7 h Sampl were taken ove the same clock-time int rval Arrows indicate value > 200 ng/ml

technical reasons sequenti l blood sampl could not be obtained from animal younger than 22 days of age The wide range f plasma GH levels in 6 - 20 day-old animals indicat howeve that GH in the rat is secreted i termitt ntly also d ring the first we ks f lif

There was no app rent sexual diff rence in the secretory patt rn of GH in rats younger than 25 days of ag In p epubert l r ts (25 30 days f age) diff rence in the s retory p ttern of GH between femal and males developed concomitantly with a diff re ce in growth te

Responsivene to GH in vitro of the rat di phragm aft acute hypophyse-ctomy (Pape V)

In rder to study the respon iveness f pe ipheral ti ue t GH ft r limination of endogenous GH the ff cts f GH in vitro on amin acid transpo t measured as the di tribution r tio f labell d AIB and protein synthesis as assessed by th incorpo ation f labelled phenylalanin into diaph agmal prot ins we e tudied at short time interv l after acut hypophyse tomy There were no dete tabl level f GH in plasma 2 6 and 24 h afte hypophysectomy Sham ope ation was also associat d with marked reduction f pl sma GH l vel for t l ast 6 h f llowing the peration but level were normal t 24 h ft r sham pe tion

At 2 6 and 24 h afte hypophyse tomy prot in ynthe i was reduced compared to control level At 2 and 6 h aft sham peration prot in synthe i was dep essed but afte 24 h there was no diff rence in the r te f p otein synthe is between the sham-oper ted and the contr l group Amino acid t an po t wa not ignificantly lt red afte sham-ope tion At 6 h aft r hypophyse tomy howeve amin id t ansport was markedly enhanced Simil observ tions have bee made in the small int tine of the t by Levitan t l (1974) who found an initi l increase in amino acid transport f llowed by de se aft hypophyse tomy I the present tudy pl sma level f GH were also low in the ham-ope ated g oup f 6 h f llowi g rgery but amino id t anspo t was not enhanced Thus the in rease in amino id transport t 6 h aft hypophyse tomy cannot be as ribed only to a lack f GH

Incubation f the hemidiaphragms with GH had no ff t on protein synthe i amino id t an po t in the diaph agms f control animals

(30 day-old females) which is in accordance with pr vi us observations (Ahrén and Hj lmarson 1968 Albertsson-Wikland and I aksson 1976) At 6 nd 24 h after hypophysectomy GH _in vitro_ stimulat d both paramete s indicating that the tis ue was responsive to th eff cts of GH a few hours aft r hypophysectomy A stimulating eff t of GH _in vitro_ on protein synthe i was also observed t 6 h aft sham ope tion The e animals h d low or undet ctabl l vels of GH in plasma foll wing surgery Th e low levels of GH in plasma for few ho rs seem to make the tis ue us ptibl to th stimulating ffects f exoge us GH on protein ynthesis

GENERAL DISCUSSION

Regulation of pisodi GH se retion

Role f monoamine It is well tablished that the monoamin NA DA and 5-HT influence GH se r tion (fo ref Reichlin 1974) but the role of monoaminergic neurotransmi sion in the regulation f pisodi GH secretion is not clear The results from the present study suggest that episodic secretion f GH in th rat i dependent upon inta t function in monoaminergic neuron Ho eove they especially indicat that ctivation of α-adrenergi receptors pl y an impo tant rol in the control of episodi GH leas in the t which i in line with independent bserv tions by Durand t 1 1977) and Martin t al (1978)

Activation f α- drenergic re ept g by admini t tion of lonidine induc GH release in therwise untre t d unanae thetized monkey (Chamber and Brown 1976) dog (Holland t 1 1978) and in man (Lal t al 1975) Moreover phent lamine an α- cept blocking gent, has been shown to inhibit GH leas in man aft insulin induced hypoglycemia exercise ginine v sop es in and ce tain tre se (f f Ma tin t 1 1978) N ad energi mechanisms therefore seem to influence GH se retion in many species If NA also pa ti ip tes in the regul tion f pisodi GH secretion in the sp i than th t i howev r not lear I man leep-associ ted GH rel ase i not aff ted by phent lamine (Luck and Glick 1971) chl rproma ine a NA and DA recepto bl ki g gent (Takaha hi t 1 1968) These finding d howeve not exclud the po ibility f I f CA in th regulation f octurnal GH se retion in man in the induced CA recepto blockade might have been only parti 1 Thu furthe tudi re equired t 1 idat the rol f monoaminergi neurot ansmis ion in the control f pisodi "phy i logi 1 GH se retion in man

In co tra t t man tre such as t auma xa ise hypogly emi and the anae the i i in rodent soci ted with an inhibition f GH secretion (f ref INTRODUCTION) indicati g important spe i diff rence in the regul tion f GH se retion In the rat tress-induced inhibition f GH se retion seems to be due to enhanced retion f GHIF (Arimu t 1 1976 T rry t 1 1976) Also f ti g which i associ t d with enhan d GH retion in man (f ref Reichlin 1974) inhibit pisodi GH se retion i the t (Pape III) an inhibition that can be eve sed

by anti serum t GHIF (Tannenbaum et 1 1978) Insulin induced hypoglycemia has however been shown to cause GH release in rats anaesth tized with gamma-hydroxybutyrate (GHB) Thi GH release can be blocked by α-HT (Blust-Pajot and Schaub 1976) It might be speculat d on that the diff rent response to unspe ific tress in the t compared t man is due t differences in the regulation of GHIF secretion and that GHB anaesthesia inhibits the secretion of GHIF in the r t

GHIF lso seems to play an important rol in the regul tion of physiological episodi GH se reti n in the rat (Ferland t al 1976) as well as in other speci (Steiner t al 1978) A po ible rol for α-adr ergic receptors could be to inhibit GHIF release In line with thi assumption i that cl nidine partially reverses *stress-induc d inhibition of GH secretion in the* r t (Edén and Modigh unpublished observations) The findi g that GH se retion till is episodic in rat t at d with antiserum to GHIF (Ferland t 1 1976) indicate th t GH se retion in the rat is also unde dynamic control f timulatory factor (GHRF) An lternative role f r α-adre rgic rec ptors could th be t timul te the rel se of GHRF In vi w of the sust ined compl t inhibition of GH secretion ft reserpine NA itself co ld po ibly be a GHRF In rats with ompl te hypothalami deaff tation GH se retion i still episodi (Willoughby t 1 1977) The hypothalamic island f these animal lack NA (Wei er t 1 1972) b t NA an re h the pituit ry and the bas l hypoth lamus from pe ipheral ource (Fuxe and Hillarp 1964) A dire t ff t of NA on the pit itary could thus xpl i diff rential effe t f treatme t with serpine and compl t hypothalade ff re tation on epi odic GH secretion Factors other than NA an however stimul te GH se retion i the r t since l trical timul tion of the VMH induces a GH rele se that cannot be blocked by phenoxybenzamine (Martin t 1 1978) Thu the pre ise ole f NA neuron in regulation of episodic GH secretion i the at remain to be established

Besides the NA neurons dopaminergic and serotonergi mechanisms might to some xtent be involved in GH regulation (for r f Marti et al 1978) Also neurotransmitters the than the monoami s may be important since agents such a picrotoxin, gamma-amino isobutyri acid antagonist and atropine have been shown to int rf re with normal pisodic GH secretion in the r t (Ma tin et al 1978) The ompl xity of the mechanisms inv lved in GH regul tion is furthe illustr ted by the finding that admi istr tion

of chl rpromazine to rethane anae thetized rat induce GH release whereas L-Dopa causes a GH suppres ion (Kato _t al_ 1973) F rther in cromegaly there is suppr ssion f lev ted GH 1 vel by L-Dop apomorphine and bromergocryptine (long-acting DA receptor agonist) These agent i duce GH release in normal ubje t The inhibitory eff ct of DA-receptor goni t on GH secretion in romegaly has rece tly been suggested to be medi t d by direct ffect on DA recept rs located in th pituit ry (f r ref Hill _t al_ 1977)

Furthe studi re thu required to elucidate the ne al control of episodic GH secretion The p sent expe iments do however indi te that pharmacological agent known t modify entr l neurotransmitt r tion can be used to f rthe lucid t these que tion Moreov r drug uch as reserpine can be used t inhibit episodi GH secretion f prolonged period f time under controlled condition Specifi inhibition of GH se retion might be valuabl in tudi on the rel tionship between pisodi GH secretion and intermedi ry metabolism F rther increased knowl dge f the mechanisms ont olling GH se tion can be helpf l in the ev luation f ne l aberr tions asso i t d with mental disord (f r ref INTRO-DUCTION)

Increased sensitivity of monoamine gi re ptors involved in GH gul tion afte ECS - po ible linical implication El trocouvul ive th rapy (ECT) is an ffective treatment in severe depre ive illness (The Royal Coll ge f Psychi tri t 1977) The mechanism( ) behind the the peuti ff t i however unknown Evidence fo long-l ting in reased sen itivity f post-synapti DA and 5-HT re ept rs fte ECT ha recently been found in be-haviour l experiment on animal (Modigh 1975 Evan _t 1_ 1976 f ref Grahame-Smith _t al_ 1978) These po t ynapti ff t f ECT might well be rel ted to the lini l ff ts f the treatment i view f the well known monoamine hypothe is f aff tive diso de (f ref Schildk t 1965)

In the present experiments GH release induced by the combined treat ment with pomorphi and lonidine admini tered 4 h aft reserpine in jection was mark dly enhanced in animal pretreated with ECS Subsequent tudi have furthe shown that GH rel ase ft combined tre tment with lonidine and 5-HTP given to serpine- ted animal i enhanced after pretre tment with ECS (Boll _t 1_ 1978) indicating that ECS lso ha e

influence on the 5-HT receptors involved in GH regulation

In view of these results it seems likely that the meas rement of GH release indu d by monoamine rec ptor agoni ts bef re and after ECT might provid model fo studying the po tsynaptic eff t of ECT in a clinical situation It may then be po sible to clarify if any changes in monoamine recept r sensitivity can be correlated to the therape tic eff ct of the treatment In this context it is also int re ting that the GH release seen aft r v ious tre tments which stimul t CA receptors has been shown to be diminished i pati nts with ffe tive disorders (Sachar 1975 Matussek et al 1977) indicating a change in CA receptor sensitivity involved in GH regul tion in patients with aff tive disorders

GH reg l tion during devel pment An increase in plasma GH levels d ri g l t f tal life and the subs quent decline has been bserved i several species uch s the rabbit (Gar ia and Geschwind 1968) sho p (Ba set t l 1971) mouse (Sihma t al 1972) dog (T ushima t l 1970) pig (Swi tek t al 1968) t (Ri ut t 1974) and i man (Kaplan t l 1976), and is p obably gene alized phenomenon Kaplan t al(1976) gge ted that the high l vels of GH i plasma du ing l te f tal lif and the e rly neonat l pe i d are due t unre tr ined stimul tion by GHRF rea hi g the pit itary by diffussion r by primitive vascular conne tions betwe the hypothalamus and the pituitary W lker t al (1977) as ibed the high levels f GH d ri g thi period f life to lack of inhibitory ontrol from the hypo- l lamus inc the levels f GHIF in the hypothalamu f ts were low ri g the first day of lif On the othe hand Ri t t (1974) bserved a marked supp ssion f GH l vel in plasma of newborn at ft r ld xpo More ver ther str ss ha bee shown to be soci ted with s upp ion f GH levels i plasma of ts 4 day ld (Stro ser d Mishle 1975) indi ti g that an inhibit ry control sy tem f GH secretion has developed at this g

I the t the ate holamines NA and DA r det t ble i the br in at 15 d y of g t tion and the high ffinity uptak p oce of NA in yn ptosomes an be demon t ated ft r 18 day f g tation (for ref Coyl 1977) Behavio l and biochemi al expe ime t have shown that NA pt rs se m to de el p very ly d ing d velopme t and beha ioural r ponses t α-adrenergi r pto goni ts an be demon t t d in ats

1 day of age (Kellogg and Lundborg 1972) Thus α-adrenergic mechanisms may be involved in GH regulation in the fetus and d i g the a rly eo- natal period resulting in high level of GH in plasma With the mat ration of ther eurotransmitt r functions in the hypothalamu an inhibit ry control system seems to develop The ge-rel ted changes in GH sec tion might thus refl ct maturation f the CNS

In the r t there is rapid increase in plasma GH levels in both femal s and mal d ring the prepubertal pe iod (Di kerman t l 1972 Ojeda and Jameson 1977) The results from the present tudy demon t at that this increase is due t in reases in peak conce t tion f GH in the GH rge and t to levated "basal level The cause f the i rease in plasma peak levels of GH during thi period i t cle Plasma l vel f prolactin show imilar increase d ring the prepubertal pe iod in the r t whereas there are no maj r changes in plasma level of LH FSH e trogens androgens (Döhl r and Wuttke 1975) It has even been suggested that GH and prolactin are important in th i iti tion f puberty (Oj d and Jameson 1977) hypothe i that might be uppo ted by the fi ding that GH treatme t to individual with isolated GH defi i n y and delayed puberty result in sexual matu tion (Sheikholi lam and Stempf l 1972) Interestingly in the growing child the se ret ry p tt rn f GH i imil to that f the prepubertal at Th GH i se reted i termittently with frequent peaks in the plasma concentration f the hormone (Pl t ick t al 1975)

Sex diff rences in the regul tion f GH se retion Gonadal t roid have been hown t influe e GH secretion In man the GH re po se t ious timuli i enhanced aft treatment with t t t on (Martin t l 1968 Illig and Prade 1970) r t gen (f ref Reichlin 1974) Also phy io- logical GH secretion i influenced by gonadal t roid A timulat ry ff f trogens GH se retion i indicat d by the higher i t g ted concent - tion f GH (ICGH) in plasma f women taking ral ont acept ves than i con trol group f women and i premenopausal women comp red po tmenop us l wo- men (Thompson t l 1974) Recently Devi t l (1977) found that ted rams had ignifican ly lowe peak concent ations f GH than control and t eatmen f he cast ted ams with ithe androgens trogens re tored pisodi GH se retion to normal

There does however not appear to be any major diff rences in the secretory pattern of GH during the estrus cycle in the rat (Saunders t al 1976 Edén unpublished observations) indicating that the ffects of gonadal teroid on GH secretion are slow in onset In mo t of the studies in which an influence of gonadal t roids has been observed the steroids were given in vivo f r prolong d pe iod of time (Martin et al 1968; Illig and P adar 1970; Dickerman et al 1972; Thompson et 1 1974) Indeed changes in brain monoamine synthe i have been ob rved after gonadectomy but not until several weeks aft r the operation (Engel t 1 1978) Moreover the DA content in the MC changes after gonad ctomy but only 4 - 5 weeks fter the oper tion (Gudel ky t 1 1977) These findings might offe an xplanation f the differen in the secretory p ttern of GH between female and male ts during the prepubertal period observed in the present experiments (Pape IV) This change in GH secretion does not seem t be rel ted to chang in irculating levels of gonadal st roids (Döhl and Wuttk 1975) Level of androgen are however highe in males than in femal s during the rly neonatal period It has been shown that femal tre ted with androgens du ing thi period of lif do not develop normal menstrual cycl s (fo ref Barr lough 1966) Such "masc linization of th br in may also be involved i the regul tion of the secretory pattern of GH Thus in the long t rm gonadal t roids may influence the ne ral mecha isms involved i the g lation of pisodic GH s etion which might expl i the sex diff e i the secretory p ttern f GH

Age- and sex- l t d diff renc i the se ret ry pattern f GH in rel tion t somati growth

The phy i logi l ignifi ance f the high ircul ting l vels f GH duri g l t f tal lif and the rly neonat l pe iod is not lear Absence of GH produce littl hange in f t l growth (fo ref Cheek nd Hill 1974) In man GH defi i ncy is soci t d with growth reta dation d ri g the first yea of life (B as l t l 1965; Rimoin t al 1968) In the r t growth ontinue t normal t f r seve al day afte hypophysectomy when the per tion i pe formed at 6 day f ge The g owth ate then gradually de li s and ompl t ly se ft r 4 week (Walko t al 1950) In this ont t it i int ti g that tis ue from normal ts gradually develop respon ivne to the timulati g ff t f xogenou GH on prot in ynthesis

and amino acid uptake d ring the first weeks of lif (Albertsson-Wikland and Isaksson 1976 N tting 1976) The ignifican e f these bservations in rel tion to the impo tance of GH for normal growth is however not clear

Pri to the prepubertal period there i no difference in body weight gain between mal and femal rats A sex diff re ce in body weight gain develop concomitantly with a mark d diff rence betwe n male and female r ts in the increase in number of nucl i in the muscle mass (Cheek t al 1971) Th development of a sex diff rence i the pattern f increase in nucl i in skel tal muscle i imil r in man (Cheek and Hill 1970) In the rat these diff rence between the sexes in somati growth seem t appear concomitantly with the developme t f diff rences in the secretory p ttern f GH between females and mal (Paper IV) If there are similar diff rence in the cr tory patt rn f GH between gi l and boy remain to be tabli hed

Other factors than GH might f course be important f the d vel p-ment f diff rence in somati growth between the sexe The pube t l growth spurt has thus been ascribed t an incre sed se retion of t sto-ste one (Thompson t l 1974) GH i howeve necessary f the normal pube tal growth pu t and it ha been sugge ted that the enhanced growth during pube ty in boy i due to the timul t ry ff t f androgen on GH secretion (Zachman and Prade 1970) E trogens on the the hand interf re with the peripheral tions f GH (Schwart t l 1969) There-f re the timulation f GH cretion by estrogen ha been thought to be ineffective due t the antagonism between the hormones in the periphery (f ref Cheeck and Hill 1974) The bserv tion in the present tudy (Pape IV) that the diff rence in growth t between mal and femal t tarts bef re the xpe t d in re se in androgen se tion in mal ts (Knorr t l 1970 Döhl and Wuttke 1975) indicate howeve that the development change in the se ret ry pattern f GH t some extent might be involved in the development f sex diff ren in somati growth and body compo ition

With increasing ge mal rat continue to grow t highe t than femal Mal t continue to se ret GH in regul pisodi pattern whereas the se retory pat rn i femal become irregul with lev t d trough l vel and lowe peak l vel Mean l vel f GH in plasma re not

lower in femal s indicating a poor correlation between mean levels of GH and growth rate (Paper IV) Also in the mouse the e eems t be a similar difference in the secretory p ttern of GH between mal s and femal s (Silms t al 1977) In man the ICGH is higher in premenopausal women than adult men (Thomp on et 1 1974) If there is a significant diff rence in the secretory patt rn of GH betwe n the sexe in man remains however to be tablished In older subj cts ale p-associated GH secretion s well s the ecretory rat f GH decrease (for ref Quabbe 1977) Also in the rat GR l vels in pl sma seem t decrease as the animal reaches it growth plateau (Dickerman t 1 1972 Edén Jan son and Fellenius unpublished observ tions)

A secretory pattern of GH with high int rmitte t peaks in plasma conce tr tion seems to be sso i ted with high growth t and prot in anabolism Hypothetically sex difference i omatic growth and body composition and the hange in body composition toward more atabolic tat with incre ing g might i part be rel t d t the se retory patt rn of GH

Rel tionship betwe n pisodi GH se tion and i t rmedi ry met boli m

The result f om the pre t study gg t an important lati n ship betwe th se r tory p tt rn f GH and it an boli ff ct It i howeve not l r if GH t dire tly on ta get t ue r if all or some of the ff ts of GH medi t d by serum facto omatomedi family of polypeptides prese t i pl sma produced in ponse to GH It i well tablished that pit it ry GH xerts dire t ff t on i t rmedi ry meta- bolism in ever l ti ue and th t these ff ts diff qualit tively f om th insulin-lik ff ts f omatomedi (Uthne t l 1974 Schw t and Goodman 1976) The mo t onvinci g evide f somatomedi s medi t f the ff t f GH ha be that GH i itro has poo ff t on til ge wh somatomedi in itro adily timul t lphat incorpo tion i t hond iti lph t i isol ted t l g a o iginally bserved by S lmon and D ughad y (1957) Somatomed ha howe not be sl own to timul t somati growth he give ivo (Uthne 1975 Thorngre t l 1977) Th lthough somatomed b ma k d ff t on int rmedi ry met bolism e l ti ue thei r le s medi t f the ff ts of GH not l ly t bli hed

Pituitary GH stimulates anabolic proc sses in several tissues (f r ref Kostyo and Nutting 1974) and it seems lik ly that also GH se ret d from the pituitary stimulates anabolic processe in pe ipher l tis ues Tissue responsiveness toexogenous GH decreases however after previous exposure to the hormone (Ahrén and Hjalmar son 1968; Goodman 1968) Rec nt-ly Isaksson t al (1978) rep rted a diurn l variation in the responsive-ness of the diaphragm muscl of 30 day-old femal rat t the timul ting effect f exogenous GH on protein synthesis Thus tissue esponsivenes to endogenous surge f GH mo t probably lso undergo change This concept is furthe support d by the finding of age relat d changes of re ponsive-ness to exogenous GH in the rat (Albertsson-Wikland and I aksson 1976 Nutting 1976) In the present tudy it was found that xogenous GH stimu-lated protein ynthesis in diaph gm muscle of rats 6 h fte hypophys-ectomy r ham ope ation indicating that low level of GH in pl sma f few hour made the ti sue sponsive t timulation by the hormone (Paper V) Moreove cent experiment in ur labo t ry have shown that exogenous GH timul t protein synthe is in 18 day-old femal t b t thi ff t lasted only f f w hours (Albertsson-Wiklund and Isak son 1978) The long dur tion f the anaboli ffect f exogenous GH report d in tudi on hypophysectomized animal might thus be due t the chronic lack f the hormone and the ff t f GH on protein synthe is in the normal animal might have sho te dur tion These findings based on in vitr experiment are in lin with the view that intermittent secretion f GH with high peak and low level between peaks may be f ruci l importance f r the anaboli ff t f GH Furthe experiment orrel ting specifi metaboli response t su g f GH in plasma re howeve required to ubstantia thi hypothe i

SUMMARY

A The role of biogenic amines in the control of growth hormone (GH) secretion was tudied in unanesthetized unrestrained adult male rats with implanted intra-aortic cann lae Administration of reserpine in a dose that effe tively blocks neurotransmission in ll type of mono aminergic neurons induced a complet inhibition of GH secretion The inhibition of GH secr tion could be prol nged with epeated administrations of reserpine Selective inhibition of cat cholamine synthe is by administration of α-methyl-p tyro ine was associated with a partial inhibition of GH ecretion Haloperidol a dopamine (DA) and noradrenaline (NA) receptor blocki g agent caused pronounced inhibition of GH secretion Administration of p-chlorophenylalanine which markedly reduced brain levels of 5-hydroxytryptamine (5-HT) and inhibited 5 HT synthesis had no effect on GH cretion Clonidine an α-rec ptor agoni t induced significant rel ase of GH i rat pr treated with re rpine whereas apomorphine a DA re ptor gonist was in ffective in this respe t It i concluded that monoaminergic neurotransmis ion is a prerequi ite for pisodic GH secretion in the r t and that α-adrenergic r ceptors are more important than DA and 5-HT rec ptors i the regulation of GH sec etion in the r t

The sensitivity of cat cholamine r ceptors involved in GH regulation were studied in adult mal t aft r pretreatment with electroconvulsive shocks (ECS) Pretreatment with ECS had no ignificant effe ts on pisodic GH cretion and GH secretion was compl tely inhibited aft r administration of reserpine in ECS pr tre ted as well as in control animals The clonidine induc d GH release after dmini tration of reserpine was mark dly enhan ed by administ tion of pomorphi e in ECS pre tr ated animal b t not in ontrol animal i dicating an incr ased sen itivity in c techolamine rec ptors involved in GH regulation ft r pretr atment with ECS It is uggest d that studies on GH secretion might be used in a linical situation to evaluate if there are change in the sensitivity of monoamine gic r epto s after electroconvulsive ther py

C

The secret ry pattern of GH was tudi d in female and mal r ts during development Plasma level declined from l tively lev ted levels t 10–15 days f age (rang : < 5–150 ng/ml) to low levels at 20 days of age (range: < 5–75 ng/ml) In 22 day-old ats there wa no significant diff rence in the secretory pattern f GH between females and male During the p pubertal period (25 30 day f age) a ignificant diff renc in the se retory p ttern f GH was ob rved In mal s (≥ 30 day ld) GH was se reted in a reg la episodi patt rn with high peaks in the concent ations of GH occurring t regular 3–4 h int rval Between peak l vel we e low and often below the d tection limit f the assay In 30 day-old femal s peak level were lower compared to 30 day-old mal In lder females (≥ 45 day) peaks occurred t irregul frequent interval and between peaks l v l were ften lev ted compared t male The sex difference in the secretory patt rn of GH developed concomitantly with a teepe in reas in the body weight gain curve in mal Hypothetically the diff ence in growth rate between female and male t afte the prepubertal period might partially be rel ted to diff rences in the se ret ry pattern f GH

D

The responsiveness f the intact r t diaph gm to exogenous GH was studi d t hort time-interval aft r hypophysectomy The pa ameters det rmined were amino acid transport as as essed by the intracellular accumul tion f the non-utilisabl amino acid AIB and prot in synthesi as assessed by the incorpo ation f labelled phenylalanine into prot in GH in vit was without ff t on any f the two parameters in normal animal Six hours aft hypophysectomy GH in vitro signifi antly timulated both amino acid t ansport and protein ynthesis indicating that the tissue respon iven to th ff t of GH we developed a few hour fter limination f endogeno GH The lts f om these experiment are interpreted in rel tion to ea li observations concerning change in ti sue responsivene to GH with time A hypotheti cal relationship between change in ti ue sensitivity and pi odi GH ecretion i discussed

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Central Monoamine Metabolism and Neonatal Oxygen Deprivation

An experimental study in the rat brain

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The present study is based on the following papers which will be referred to by their Roman numbers

I Hedner T and Lundborg P (1978): Regional changes in monoamine synthesis in the developing rat brain during hypoxia
Submitted for publication Acta physiol scand

II Hedner T and Lundborg P (1978): Catecholamine metabolism in neonatal rat brain during anoxia and recovery
Submitted for publication Acta physiol scand

III Hedner T and Lundborg P (1978): Serotonin metabolism in neonatal rat brai during anoxia and recovery
Submitted for publication Acta physiol scand

IV Hedner T Lundborg P and Engel J (1978): Biochemical and behavioral changes in 4 weeks old rat brain after neonatal oxygen deprivation
Submitted for publication Pharmac Biochem Behav

V Hedner T and Lundborg P (1978) Survival of neonatal rats during hypoxi after central monoamine receptor stimulating agents
Submitted for publication Pharmac Biochem Behav

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Introduction

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INTRODUCTION

1 1 Perinat l asphyxi

Oxygen deprivation is one of the most common and damaging conditions affect ing the human br i While erebral oxygen lack I a catastrophic event in the adult It is somewhat less In th immature organism with a dev loping nervous system This is due to a higher resistance t hypoxi anoxi and ischemia (Jilek et al 1970)

Clinical investigations have shown that there exists an inverse corr lation between symtoms of perinatal asphyxi as measured by Apgar core of the newborn and the elative frequency of neurologi abnorm lities of the child (Drage and Berendes 1966) Severe intrapartum asphyxi has further been associated with low ering f mean IQ scores at 4 years of age (Berendes 1967) It has also been suggested that early childhood behavioral disorders such as the so called minim l brain dysfunction syndrom I som cases may have perinat l oxygen deprivation as an underlying factor (Clements 1966 Towbin 1971 Handford 1975 Wender 1974)

Experimental animal studies hav demonstrated that different forms f severe oxygen deprivation such as hypoxi anoxia or ischemia have one common neuropathological denominator (Brierly t al 1973) These neuronal changes found I many parts of the brain includes microvacuolation swelling of mitochondria enlargement f extracellular space swelling of endoplasmi reticulum Golgi changes loss and lumping of synaptic vesicles and also swelling of astrcytes (Spi lmeyer 1922 McG e-Russel et al 1970 Brierly et al 1973 Salford t al 1973) However not ll egions of the brain are equally involved but neuronal alterations are found in areas which appear to exhibit selective vulnerability" to oxygen deprivation (Brierly et l 1973) I the adult animal invol ement is generally found I the cerebral cortex hippocampus amygdaloid nucleus cerebellum and the brain stem The spinal cord is resistant whil involvement of the thalamus

striatum pallidum and subthalamic nucleus is variable (Brierly et al 1973)

In the neonatal period oxygen deprivation seems to result in two different patterns of brain damage this d pending upon whether the insult is a brief acute episode of total asphyxia or a more prolonged partial disruption of the oxygen supply (Myers 1972) A total asphyxia up to 20 min in the monkey induces a highly reproducible pattern of injury involving structures in the brain stem The cerebr l cortex and the basal ganglia are damaged very late in the process or not t all I contrast to this a prolonged partial asphyxia results in morphologic changes involving hemispheral structures and occasionally the basal ganglia (Myers 1972) The type of injury seen after prolonged asphyxia resembles the pattern of injury seen typically in the human eonate in contrast to the injury found after a short period of total oxygen deprivation

Under normal circumstances the preponderant portion of cerebral energy is supplied through oxidative reactions in which oxygen is required As a consequence oxygen depr vation causes rapid and profound alterations in metabolic activity Cerebral function thus ceases after brief periods of oxygen deprivation mainly due to the high energy demands of the brain During oxygen insufficiency anaerobic glucose metabolism prov des the principal energy source and survival is rel ted to energy production (see Cohen 1973) The decreased survival time during hypoxia or anoxia has been related to cardiac impairment as well as cerebral invol ement (Brockman and Jude 1960 Miller and Myers 1970) In the imm ture anim l the resistance is greater to oxygen deprivation than in the adult (Jilek 1970) One explanation to thi find ng may be a lower cerebral metabolic rate (Berlet 1976) The increased capacity for anaerobic metabolism in the neonatal animal may also contr but to the increased resistance as inhibition of the glycolytic cycle decreases sur ival and adm nistration of glucose prior to anoxia increases survival (Himwich et al 1942)

All energy produced i the brai under norm l ci cumstances result from three interrelated phases of glucose metabolism· (1) glycolysis (2) tricarbolic acid cycle (Krebs cycle c tr c acid cycle) metabolism (3) elect on transport system (see Cohen 1973)

Glycolys s proceeds either aerobically or anaerobically although oxygen is not equired for the i itial degradation of glucose The endproduct of aerobic glycolysis is pyruvat wh ch enters into oxidative metabolism Small amounts of pyruvate is however reduced to lactate and i the absence of o ygen all pyruvate formed

is reduced to lactate When oxygen is present glycolysis is inh bited (Past ur ef fect) while in its absence there is augmentation of glycolyti activity with ncreas ed formation of lactic acid (see Cohen 1973) Thus increasing concentrations of cerebral pyruvate as well as lactate are noted with increasing anoxia (Lowry et al 1964 Vannucci and Duffy 1976) The intracellular accum lation of lactat produces a marked local acidosis (Kaasik et al 1970) Initially the rise in lactate concentrations is rapid while the levels diminish slowly after anoxia (Vannucci and Duffy 1976) In contrast to this the levels of glucose and pyruvate increase markedly immedi tely following anoxi before a gradual decline is noted (Vannucci and Duffy 1976)

Pyruvate formed through glycolysis is further metabolised through the tricarboxylic acid cycle It is decarboxylated to acetate which reacts with coenzyme A to form acetyl coenzyme A This compound condensates with oxalacetat to form citric acid (see Cohen 1973) Although lterations i tricarboxylic acid m tabolism and its involved enzymes have been stud ed minimally the activity of succin l dehydrogenase malic dehydrogenase and glutami dehydrogenase is known t decrease during severe oxygen depri ation (MacDonald and Spector 1963 Spector 1963 a Spector 1963 b)

Reductions of carrier compounds withi the electron transport system occur during oxidative tricarboxyli acid cycle activity The educed carriers are e oxidized and the lectron is given up for passag to the next compound in the chain At the end of the electron passage ther is combination with oxygen and water is formed The eactions of the carrier compounds i the electron transport system are the major sources of energy formation

The energy deri ed from these various sources esides in th high energy com pound adenosine triphosphate (ATP) Energy for cerebr l processes is leased from ATP through dephosphorylation of the terminal phosphate and adenosine diphos phat (ADP) and inorgani phosphat is formed Nervous system energy req ire- ments vary with neural acti ity and do not compl tely parall l supply Transfer of the terminal phosphate of ATP t creatine to form phosphocreatine allows for some storage of energy i the l tter compound Under conditions of high demand the high energy phosphat is returned to ADP thus forming ATP (see Cohen 1973)

Oxygen deprivation interferes with high energy phosphate ynthesis in the elec tron transport system Under these ci cumstances ther is a apid decrease i the concentrations of energy-rich compounds (Vannucci and Duffy 1976) Generally

phosphocreatine concentrations change prior to alterations in cerebral ATP due to its function as a storage compound (Lolley and Samson 1962 Vannucci and Duffy 1976) Following anoxia the levels of these energy-rich compounds are rapidly restored (Vannucci and Duffy 1976)

Significant alterations in cerebral hydrogen ion concentrations have been observed with oxygen deprivation During ischemia and anoxia the pH falls (Thorn and Heitmann 1954 Vannucci and Duffy 1976) and t has been suggested that the early effect of anoxia on metabolic systems is due largely to increasing acidity (Hall 1966)

Anoxia is also associated with changes in sodium and potassium concentrations and these changes may exert secondary effects on cerebral metabolism With decreasing O_2-tensions extracellular sodium decrease while potassium concentrations increase in the young as well as in the adult brain (Meyer et al 1965 DeSouza and Dobbing 1973 Hansen 1977)

Alterations i neurotransmitter metabolism other than monoamine metabolism have been studied minimally However available data indicate that cerebral acetyl *choline content and synthesis is depressed during oxygen deprivation* (Welsh 1943 Gibson and Blass 1976) In contrast to this the levels of the inhibitory neurotransmitter gamma amino butyric acid (GABA) increase during ischemia i the neo-*natal as well as in the adult brain* (Lust et al 1975 Jilek 1970)

Although the cerebral metabolic response to oxygen deprivation is extensive *neurological deficits precede the depletion of energy-rich phosphates* (Siesjö and Zwetnow 1970 a Siesjö and Zwetnow 1970 b Duffy et al 1972) Behavioral symptoms i animals as well as in man are found at an early stage during oxygen deprivation (Meier 1971) In general i man the complex higher mental processes are the first to suffer from the oxygen deficiency whereas the simpler and more *peripheral processes are unaffected until most extreme oxygen deprivation* is reached Thus symptoms include e g motor incordination impai ed attention and concentration i tial euphoria followed by depression and irritability (Bagby 1921 Dunlop 1918 Johnson and Pascal 1920 Lawson 1923) Similarly animal experiments show that acute exposure to low oxygen concentration causes deterioration in open field behavior (Hurder 1951 Thomson and Pryer 1956 Vacher and Miller 1968) and operant behavior (Seitz and Keller 1940 Hurwitz et al 1971) In the adult clin cal and experimental studies demonstrate longterm behavioral sequelae (depression and amnesia or impairment of locomotion and isual discrimination respectively)

after profound oxygen lack (Allison 1950 Nicholson et al 1970) In contrast to the adult form of deprivation the likelihood of oxygen deprivation during fetal and neonatal life is high and has been a matter of considerable research interest Thus lear ing impairment and early childhood behavioral disorders have been asso- iated with periods of perinatal oxygen lack (Berendes 1967 Towbl 1971 Wender 1974)

1 2 Central monoaminergic neurons

1 2 1 Distribution

The cell bodies of the monoamine containing neurons are with few exceptions located in th lower brain stem i e mesencephalon pons and medulla oblongata (see Fuxe et al 1970) Some short noradrenaline (NA) and 5-hydroxytryptamine (5-HT) neurons lso have their nerve terminals within the lower brain stem Other NA and 5-HT neurons the bulbospinal group form descending pathways with nerve terminals in th ventral dorsal and lateral horns of the grey matter i the spinal cord The major part of the monoamine neurons form ascending pathways from the lower brain stem Among these NA neurons form a entral pathway with ter minals in the diencephalon e g hypothalamus and the septal area A dorsal NA pathway originates from nerve cells in the locus coeruleus terminating in the neo- cortex hippocampus and cerebellum and several other areas f th brai wher it overlaps with the entr l pathway (see Fux et al 1970)

The major pathways of dopamine (DA) neurons ascend from the substanti igr to the corpus striatum (nigro-neostriat l pathway) and from ther cellgroups in the mesencephalon to the nucleus accumbens and other parts of the limbic forebrai (meso-limbic pathways) (see Fuxe et al 1970)

The 5-HT neurons form pathways m l ly arising from cell bodies f the raphe nucleus in the mesencephalon ascending in the medial forebrain bundl terminating i in the limbic forebrain the hypothalamus the corpus striatum and the neo- cortex (see Fux et al 1970)

Recently evidence for the existence of adrenaline neurons in the CNS hav emerged (Hökfelt et al 1973 Hökfelt et al 1974) These neurons have their c ll bodies i the lower brai stem and their terminals i th spinal cord the lower brain stem the locus coeruleus and the hypothalamus

1 2 2 Synthesis storage and release

The first step in the synthesis of catecholamines (CA) is the hydroxylation of the amino acid L-p-tyrosine to L 3 4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (Blascho 1939 Nagats et al 1964) This highly specific step (Udenfriend et al 1965) appears to be the rate-limiting reaction in the synthesis of CA under normal conditions (Levitt et al 1965) In the adult rat tyrosine hydroxylase appears to be saturated to about 75% (Carlsson and Lindqvist 1978) This reaction seems to occur in the cytoplasm of the catecholamine neurons (Laduron and Belpai e 1968) Tyrosine hydroxylase is competitively inhibited by α-methyltyrosine and a few other tyrosine analogues (see Udenfriend 1966) The next step the decarboxylation of L DOPA to DA is catalyzed by aromatic L-amino acid decarboxylase a widely distributed and much less specific enzyme (Holtz et al 1938 Rosengren 1960 Lovenberg et al 1962) This reaction is supposed to occur in the cytoplasm (Hagen and Cohen 1966, Molinoff and Axelrod 1971) Aromatic L-amino acid decarboxylase can be inhibited by e g 3-hydroxybenzylhydrazine (NSD 1015) (Carlsson et al 1972) NSD 1015 can be used to study the in vivo activity of tyrosine hydroxylase (and tryptophan hydroxylase) as the intermediate amino acids (DOPA and 5-hydroxytryptophan respectively) accum late in a linear manner the first 30 min after decarboxylase inhibition These intermediates seem to accumulate intraneuronally and are not appreciably metabolized or transported from the region studied (Carlsson et al 1972) The enzyme DA-β-hydroxylase which is localized in the storag granules (K rchner 1959) is respons ble for the conversion of DA to NA (Levin et al 1960) This enzyme is far from specific and catalyzes the hydroxylation of several other phenyletylamine derivates to their corresponding ethanolamines (Carlsson and Lindqvist 1962 Carlsson and Lindqvist 1967)

The synthesis of 5-HT proceeds from the amino acid precursor L tryptophan This amino acid is converted to L 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Udenfriend et al 1956) and this eaction appears to be the rate-limiting tep i the 5-HT synthesis The enzyme which probably is specific for L tryptophan appears to be about half-satur ted with its amino acid substrate (Carlsson and Lindqvist 1978) This enzyme (as well as tyrosine hydroxylase) utilizes molecular oxygen as a co-substrate (Costa and Neff 1970 Daly t al 1968) The next step which forms 5-HT i the decarboxylation of 5-HTP by the aromatic L-amino acid decarboxylase (ide supra)

The newly formed transmittor amines synthetized i the monoaminergic neurons are largely taken up and tored in granules located in the enlargements (varicosities) of the nerve terminals The granular storage of these amines is probably essential for the release of transmittor induced by nerve impulses (see Malmfors 1965 Häggendal and Malmfors 1969 Andén et al 1969 W iner 1970 Dahlström et al 1973 Smith 1973) Different mechanisms are present for terminating the actions of the released transmitters Probably the most import nt under physi logical conditions is reuptake into the presynaptic nerve terminals followed by catabolism or reuptake into granules (see e g Andén et al 1969 Carlsson 1970 Iversen 1970) The CA are enzymatically inactivated intraneuronally by monoamine oxidase (MAO) and after release into the synaptic cleft mainly catabolized by the enzym catechol -O-methyl transferase (COMT) (see Jonason 1969) 5-HT is chiefly catabolized both inside and outside the 5-HT neurons vi MAO (see Blaschko and Levine 1966)

1 2 3 Ontogenesis

Th ontogenesis of the central nervous system is a complex process invol ing cell multiplication cell migration outgrowth f neuronal processes and synaptogenesis (Himwich 1970) Brain growth i not a uniform process but rather an ir regular maturation of certai populations of neurons and brai regions As a consequenc there is a constantly shifting pattern of neuronal relationships during the developmental period (see Coyle 1973) The developmental patterns of whol brai monoamine contents and enzymes inv lved i their synthesis and metabolism is highly species dependent Whil the guinea pig and chick who are relatively mature at birth have nearly adult brai contents of monoamines the rat mouse cat rabbit and man who ar born relativ ly immatur have correspondingly low brain amine levels at birth (see Thornburg and Moore 1976) From histological biochemical and physiological studies it is appearent that the central monoamine neurons are present at a very early stage i the mammal brai (see Thornburg and Moor 1976) Al though these neuronal systems develop mechanisms for ynthesis and storage at a very early stage during ontogeny the brai levels of DA NA and 5-HT i the rat increase progressi ly with ag and do not reach ad lt levels until several weeks after birth or as in the case of DA not until adult lif (see Thornburg and Moor 1976) This progressive increase i central monoamine neurotransmittor lev ls with age is probably a consequence of the centrifugal outgrowth of axons and ter minal from the cell bodies in the lower brain stem (Loizou 1969 Loizou 1972)

The maturation of the DA NA and 5-HT content in the rat brain progresses in a d stinct caudal to rostral direction (see Thornburg and Moore 1976) Analysis of amine content in the whole rat brain suggests that the sequence of development of DA is somewhat delayed compared to that of NA and 5-HT (see Thornburg and Moore 1976) Available data also indicate that both NA and 5-HT containing neurons become functional (I e impulse-conducting) at an earlier age than DA -containing neurons (Kellogg and Lundborg 1972 a Kellogg and Lundborg 1973)

The acti ities of the fi st and rate limiting enzymes of the catecholamine and indoleamine pathways tyrosine hydroxylase and tryptophan hydroxylase respectively have been detected in the rat brain during late gestation (Schmidt and Sanders Buch 1971 Coyle and Axelrod 1972 a Coyle and Axelrod 1972 b) The increases in enzyme acti ities during development closely parallel the levels of their corresponding amines *(see Thornburg and Moore 1976) Analysis of the regional distr'b-* ution of tyrosin hydroxylase and tryptophan hydroxylase in the rat brai indicates that the maturation of these enzymes also proceed in a caudal to rostral direction (Deguchi and Barchas 1972 Coyle and Axelrod 1972 b) Thus adult values for the respective enzymes were att ined much earlier in the brai stem compared to e g the corpus striatum and hemispheres (Coyle and Axelrod 1972 a Coyle and Axelrod 1972 b Hedner and Lundborg in manuscript) The development l pattern of aromat ic L-amino acid decarboxylase resembles that of many other brain enzymes I e a sharp ise i enzyme acti ity at birth followed by a slight fall nd then a grad ual rise to ad lt activity at about 6 week of age (Bennet and Glarman 1965) The *matur tion of the two degradative enzymes MAO and COMT proceed in a dishar* monious way MAO acti ity in the rat brain increases after bi th to attai adult values at the third of fourth postnatal week Regional studies lso suggest a caudal to rostral patter of development (Bennet and Glarman 1965 Porcher and Heller 1972) In contrast to this COMT is similar in all bra n regions and equal or greater *than adult acti ity throughout the postnat l period (Porcher and Heller 1972)* The development of a storage mechanism I the catecholamine neurons seems to develop in parallel to the endogenous catecholamine content in the at brain The specific uptake mechan sm at th neuronal membrane however occurs earlier than the development of the intraneuronal storage capacity (Coyle and Ax lrod 1971) The *developmental aspe ts f the high affi ity neuronal membrane uptake system for* 5-HT am ins largely to be studied

Yet little is known about the dynamics of the monoamine neuronal populations

In the CNS t various stages of development However tudies of drug actions at DA NA and 5-HT synapses ind cate that the biochemical and functional differentiation precedes complete morphological differentiation (Lai ou 1971 Kellogg and Lundborg 1972 b Lundborg and Kellogg 1974)

1 3 Aim of the present study

The principal aims of the experimental work in thi thesis were:

- to study the effects of oxygen deprivation on catecholamine nd serotonin metabolism in the neonatal rat brai
- t study the brain monoami e m tabolism after immediate and late ecovery from neonat l oxygen deprivation
- to study th effect of acute hypox on tyrosine hydroxylase and tryptophan hydroxylase activity in different brain regions i the neonatal and developing rat
- to study the correlation between the persistent biochemical and functional changes in the brai after sever neonatal oxygen deprivation
- to evaluat the ffects of various monoamine stim lating agents *on the survival time of neonatal rats during hypoxia*

METHODOLOGY

2 1 Subjects

Sprague-Dawley ats were used throughout the studies Pregnant rats (16th–18th day of gestation) were obtained (Anticimex Stockholm) and housed i the department *in separate cages The animals were kept under regulated dark light* conditions (light period 6 a m 6 p m) and the tim of delivery was noted within 12 h Experiments wer performed on the offspring t 1 4 14 and 28 days of postnatal age All experiments started around 4 h after the onset of light

2 2 Induction of oxygen deprivation

Hypoxia or anoxia was produced by passing oxygen and/or nitrogen gas (Aga

Sweden) through two gas flow meters (The Matheson Co East Rutherford N J) at a rate of 4 l/min into a 22-litre plastic cage for periods of 20 min 30 min 4 5 h or longer The gas or gas mixture was let through the cage via inlet and outlet valves Rubber gloves were mounted to the sides of the box to allow handling of the animals without disturbing the equilibrium of the system by opening the box Experiments were performed after a 30 min equilibrium period Control animals were kept in a similar open box exposed to room air The cages were kept on a preheated (35-36°C) table and room temperature was at 27°C At various intervals during the studies the oxygen content of the gas mixture was analysed

In some studies (II III IV) the I font rats were returned to normal atmosphere at various intervals before decapitation while in other experiments (I II III) the animals were decapitated immediately after the exposure to hypoxia or anoxia 30 min befor sacrifice all animals in which the brain was analyzed for monoamine precursors were injected with NSD 1015 100 mg/kg

2 3 Substances used

Apomorphine (Sandoz)
Clonidine HCl (Boehringer Ingelheim)
L-DOPA (Astra)
L 5-HTP (Hässle)
NSD 1015 (Synthetized i this department)

2 4 Brain dissection

The ats were killed by decapitation The whole brain (without olfactory lobes) was quickly removed and dissected on an ice-cold glass plate into the following parts: (1) Striatum including corpus striatum and limbic forebrai (2) hemispheres including hippocampus (3) diencephalon (midbrain) and (4) lower brain stem Cerebellum was identified and discarded Dopamine is the predominating catecholamine in part (1) and noradrenali in parts (2) (3) and (4) Most of the 5-HT contai ing nerv ell bodies are found in part (4) For details of the dissec tion procedure see Carlsson and Lindqvist (1973)

Immediately after the dissection procedure the brain parts were frozen on dry ice In the 4 days old ats 10 parts were pooled in the 14 days old 4 parts and in the 28 days old 2 parts were pooled and weighed In the experiments where the

whole brain was analyzed (II III IV) 4 brains were pooled from 1 day old animals while the br ins of the 28 days old animals were analyzed separately The brain samples were stored I a freezer at 70°C in no case for more than 2 months

2 5 Biochemical determination of monoamines and their precursors

After thawing the pooled brain parts of the *whole brains* were *homogenized* in 10 ml of 0 4 N perchloric acid containing 5 mg $Na_2S_2O_5$ and 20 mg EDTA The homogenates were centrifugated at about 10000 x g for 10 min at 0°C and the supernatant purified on strong cation exchange column (Dowex 50-X-40) (Atack and Magnusson 1978) The separated amines wer analyzed spectrofluorometrically according to the following methods (see Atack 1977):

Tyrosin (Waalkes nd Udenfriend 1957 Kehr et al 1972)
DOPA (Kehr et al 1972)
Dopamine (Atack 1973)
Noradrenaline (Bertler et al 1958)
Tryptophan (Bédard et l 1972 Kehr et l 1972)
5-HTP (Atack and Lindqvist 1973)
5-HT (Atack nd Lindqvist 1973)

2 6 Behavioral assays

The acq isition of a conditioned avoidance esponse was measured by means of a two-way shuttle-box (IV) The rats were trained to avoid an electric shock (unconditioned stim lus UCS) with the sound of a house buzzer as warning stimulus (conditioned stim lus CS) In each trial the CS was presented for maximally 10 sec followed by the CS plus the UCS for another 10 sec A conditioned avoidanc response was defined as a crossing through the opening within 10 sec after the presentation of the CS An escape was a cross within 10 sec after the shock (UCS) had been delivered Prior to the first training session the rats were llowed to adapt to the shuttle-box for 30 mi T aining sessions lasted for 20 min and consisted of 20 trials

2 7 Statistical methods

For statistical evaluation Student's t-test or a non-parametric multivariate analysis

(Mantel et al 1970) was used The level of significance was chosen as $p = 0.05$

RESULTS AND DISCUSSION

3 1 Acute effects of oxygen deprivation on catecholamine metabolism (I II)

The susceptibility of the adult and the neonatal brain to hypoxia is generally greater than other organs due to its high demand for oxygen and continuous energy supply In the adult rat brain environmental oxygen concentrations of 7% or higher cause no apparent alteration in energy metabolism (MacMillan and Siesjö 1971) yet exposure to this degree of hypoxia produces behavioral disturbances (Hurder 1951 Brown et al 1975) Recent studies on adult rats from these laboratories have correlated the behavioral disorders caused by hypoxia to alterations in the metabolism of the monoamine neurotransmitters in the brain (Davis and Carlsson 1973 b Brown et al 1975)

Investigations on neonatal (1 to 4 days old) and developing animals during hypoxia have demonstrated a marked decrease in the activity of tyrosine hydroxylase as measured by DOPA accum lation after decarboxylase inhibition by NSD 1015 (Hedner et al 1977 a Hedner et al 1977 b Hedner et al 1978) In the present study complete anoxia for 20 min caused a reduction of DOPA accumulation by about 25% i the whole brain of the neonatal animal which is in parallel to the reduction found i 1 28 days old rats after 30 min of 6–12% hypoxia (Hedner et al 1977 a Hedner et al 1977 b Hedner et al 1978) In general our previous studies have shown that tyrosine hydroxylase is markedly inhibited already at 12% environmental O_2, and as a rule tyrosine hydroxylase activity decreased to about the same extent during the various hypoxic levels (6–12% O_2) at all ages studied (Hedner et al 1978)

However i our previous studies when the period of hypoxia was extended from 30 min to 2 and 6 h a restitution of tyrosine hydroxylase activity was found in the neonatal but not in the adolescent animals (Hedner et al 1977 b) In the adult animals similar adapti e changes occur when the exposure to hypoxia is prolonged further (36 h 10% O_2) (Dav s 1975) This adaptation of brain catechol amine synthesis to hypoxia appeared to correlate with adapti e changes in brain t ssue oxygen rather than any chang in the intraneuronal regulation of amine synthesis A more chronic exposure to a moderat hypoxic environment (12 8% O_2)

from birth until adulthood have shown that the activity of catecholamine synthetizing and catabolizing enzymes decrease in some brain areas and increase in others during this form of stress (Vaccari t al 1978 a) The differences in response to a short (30 min) and a mor prolonged hypoxia (6 h) found in the neonatal but not in the older rats may be caused by a variety of factors such as e g changes over time i tissue oxygen tension pH changes shifts i the hemoglobi dissoci tion curve late alterations in energy m tabolism etc Th quicker restoration of enzyme activity during prolonged hypoxia in th neonatal animal may indicate that the catecholamine neuronal system in the neonatal rat brain may be mor resistant to oxygen lack compared to th ad lt brain

As the dev lopment of tyrosine hydroxylas activity in different brain regions does not proceed i a harmonious way studies were performed to investigate possi ble regional differences in sensitivity t hypoxia i th neonatal animals there were significant decreases during 30 mi 6% hypoxi i ll brain regions studied except for the hemispheres At 28 days of ag DOPA accumulation after NSD 1015 decreased markedly in ll egions at 14 days f age a significant decrease was noted i hemispheres and midbrai but not i striatum and brain tem It is not like ly that th differences in response to oxygen deprivation between different ages and br i egions reflect qualitative lterations i tyrosine hydroxylase The lack of effect i th hemispheres region at 4 days f ag may be due to quantitative factors such as the lack of outgrowth f techolamine neurons to thi region at this stage of postnatal development (Loizou 1971) However th differ nces in response could lso be the esult of other important maturational events occurring i the rat brai during development

The esults do not seem t talk i favour of differ nces i sensibility to oxygen lack between NA and DA neuronal pathways as a principally NA-storing area (hemispheres) and a principally DA-storing area (striatum) seems to be equally f fected

Tyrosine levels wh ch were measured i parall l to DOPA did in general not change during hypoxi and thus no appearant correlation between the levels of this precursor amino acid and the rate of tyrosine hydroxylation was observed during short period of anoxia or hypoxi

Th endogenous levels of the neurotransmitters DA and NA did not change in the neonatal rat brai during 20 min of anoxia Thi may be expl ined by an i hibition of firing of catecholamine neurons during oxygen deprivation or a decrea

degradation of neurotransmitters Studies performed on the adult rat brain during hypoxia have given support for both these explanations (Davis and Carlsson 1973 Brown et al 1975) Significant decreases in the endogenous levels of DA and NA in the adult brain during hypoxia have however previously been reproted (Strupfel and Roffi 1961 Debijadi et al 1969 Hurwitz et al 1971) In all of these studies however several hours to days of exposure were used to produce those changes Thus before such changes in the actual amine levels can be identified pronounced alterations in their production and metabolism can be demonstrated at more subtile changes in oxygen availability Apart from the extensive effects of hypoxia on tyrosine hydroxylase the third enzyme in the catecholamine synthesis pathway i e DA-β-hydroxylase is also partly inhibited by hypoxia at least in the adult rat (Brown et al 1975) Furthermore the degradation of catecholamines in the adult rat brain has been found to be affected since the monoamine oxidase is partly inhibited by hypoxia (Brown et al 1975) The second enzyme involved in the catecholamine degradation i e COMT or the other non-oxidative steps in the catecholamine pathway do not seem to be influenced by acute hypoxia (Brown et al 1975)

3 2 Acute effects of oxygen deprivation on 5-HT metabolism (I III)

In accordance with tyrosine hydroxylase the enzyme tryptophan hydroxylase requi es molecular oxygen for the synthesis of the ew hydroxyl group of its neurotransmitter product I ltro and in vivo studies suggest that the enzyme is not ful ly saturated with oxygen under normal conditions and an increase in enzyme ac ti ty occurs with increased availability of oxygen (Green and Sawyer 1966 Davis and Carlsson 1973 a) Using an in vivo technique developed in this laboratory (Carlsson et al 1972) a decrease in enzyme activity could also be demonstrated i the adult rat brain during hypoxia (Davis and Carlsson 1973 a Davis and Carlsson 1973 b Davis et al 1973)

Also in the neonatal animals we could demonstrate a decreased accumulation of brain 5-HTP after NSD 1015 the intermediate product in the indoleamine synthesis pathway after oxygen deprivation (Hedner et al 1977 a Hedner et al 1977 b Hedner et al 1978) Although a marked inhibition of tryptophan hydroxy lase activity after 12% hypoxia occurred in all age groups studied the effect appeared to be somewhat less pronounced in the early postnatal period than later in development (Hedner et al 1977 a) \ hen the environme tal oxygen concentra-

tion was lowered from 12% to 8% and 6% a further decrease in enzyme activity was noted in only a few cases (Hedner et al 1978) When however the period of 12% hypoxia was prolonged to 6 h this resulted in a normalisation of enzyme activity or in the neonat l rats even a increased activity compared to controls (Hedner et al 1977 b)

Similar results have been obtained i the adult rat brain during exposure to similar levels of hypoxia for 36 h (Davis 1975) Exposure to chronic oxygen defi ciency (high altitude 12 8% O_2) in developing rats from birth to adulthood results in a disturbed brain neurotransmitter synthesis The activity of tryptophan hydroxylase increased in some discrete brain areas and decreased in others during development (Vaccari t al 1978 a)

I the adult rat br in tryptophan hydroxylase has been shown to be dependent on the arterial oxygen tension (Davis et al 1973) At arteri l O_2 tensions below 60 mm Hg there was a decreased accumulation of cerebral 5-HTP after decarboxylase inh bition with NSD 1015 The decrease in tyrptophan hydroxylase activity during acute hypoxia was noted before detectable changes in cerebral energy metabolism were evident (Davis et al 1973) Thus tryptophan hydroxylase appears to compet poorly with mitochondrial enzymes for available oxygen

I the study on regional brain areas there was a marked decrease i 5-HTP accumulation fter NSD 1015 in all brai regions (hemispheres striatum midbrain and brai stem) at all ages studied The relative decrease i the different brain areas appeared t be similar for the various ages studied The effect of hypoxia on 5-HT synthesis in these brain areas seemed to be mor uniform than th ffect of the synthesis of the brain DA and NA under identical conditions The possibility that NSD 1015 induces changes in c ll lar energy production thereby making the tryptophan hydroxylase enzym mor sensitive to hypoxia cannot be excluded ince slight changes in erebral energy metabolism do occur after NSD 1015 admi i tration (Davis et l 1973) Results similar to ours have however been achieved by means of other methodology (T icklebank et al 1976) When 5-HT synthesi rate was estimated by means of MAO inhibition decrease in synthetic rate by approx imately 40% was found i neonatal rats asphyxiated to the point f death Although different experimental models were used the esults ar i agreement with our findings

The actual levels of the neurotransmitter precursor tryptophan wer gener lly not affected during a short period of oxygen deprivation (20-30 mi) If however

the exposure to hypoxia is prolonged tryptophan levels have been found to increase and exceed control levels in both neonatal (Hedner et al 1977 b) and adult animals (Davis and Carlsson 1973 b) Tryptophan is bound to album n in the plasma and the degree of binding to albumin is very sensitive to minor changes in pH in the range of 7 0 8 0 (McMenamy and Oncley 1958) Changes in the albumin binding of tryptophan during hypox c states could thus influence the tryptophan hydroxylation since the enzyme is not saturated with tryptophan under normal conditions in vivo (Carlsson et al 1978)

No changes n the endogenous levels of 5-HT were found in the neonatal animals during 20 min anoxia Studies on adult animals have demonstrated that besides the decrease in the synthesis of brain 5-HT during hypoxia there is also a decrease in the degradation due to an inhibition of the major degradative enzyme MAO (Davis and Carlsson 1973 b) Thus hypoxia appears to be one in a group of conditions which in the brain may alter 5-HT metabolism without affecting 5-HT levels

3 3 Monoamine metabolism after recovery from anoxia (II III)

Several metabolic esponses occur after acute anoxia in neonatal animals Energy stores which decrease during anoxia are normalised or even increased during the recovery phase (Vannucci and Duffy 1976) In a similar way we found increased levels of DOPA and 5 HTP in the one day old animals after decarboxylase inhibition with NSD 1015 immediately after the exposure to 20 min 100% N_2 The increase i tyrosine hydroxylase and tryptophan hydroxylase activity was of a transient nature and 2 h after the insult enzyme activity had returned to control levels No further appearent changes in the activity of these synthetizing enzymes were noted during the following 48 h after anoxia

In parallel with the increases in tyrosi e hydroxylase and tryptophan hydroxylase activity observed the level of the precursors tyrosine and tryptophan were increased in the immediate postanoxic phase As the rate-limiting enzymes tyrosine hydroxylase and tryptophan hydroxylase are not fully saturated with their respective precursor amino acids under normal conditions (Carlsson and Lindqvist 1978) the increased levels of DOPA and 5-HTP after anoxia may i part depend on the increased availability of the precursors

In contrast to the changes i enzyme activity no major changes in the endogenous levels of DA and NA were observed during the recovery period The endogenous levels of 5-HT d'd however increase in parallel with the increases in

enzyme activity and precursor availability These results are not surprising since one major factor i the control of 5-HT synthesis is the availability of tryptophan This close correlation between the precursor amino acid and the neurotransmitter product does not seem to be present for DA and NA

3 4 Late effects of oxygen deprivation on monoamine metabolism (IV)

Exposure to hypoxia or anoxia is a complex form of stress in the neonate inducing both sympathetic hyperactivity (Jones and Robinson 1975 Lagercrantz and Bistoletti 1978 Vaccari t al 1978 b) and adrenocortical hyperfunction (Vaccari et al 1978 b) A considerable body of evidence supports that oxygen deprivation during development may induc a wide range of persistent neurologic and neuroendocrinologi alterations in the brain (Towbin 1970 Myers 1972 Simon and Voli er 1976)

The outgrowth of the DA NA and 5-HT containing neuronal systems originate mainly from some areas i the brain stem (ide supra) As these brain regions seem to be vulnerabl to perinatal asphyxia (Myers 1972) some behavioral disorders of early childhood (e g infantil autism) have been corr l ted with alterations in monoamine metabolism (Wender 1972)

The newborn rats subjected to 20 mi anoxia or 4 5 h 6% hypoxia in our experiments did not becom spastic or display any igns of subsequent gross motor impairment nor did the br in weight differ between th anoxia or hypoxia group and the control group at 28 days of age Previous studies hav however indicated that neonatal asphyxi may esult in a retardation of both body and brain growth (Vannucci and Duffy 1976 Simon and Volicer 1976)

In our investigations we have found that neither total anoxia for 20 mi or 6% hypoxia for 4 5 h during the neonat l period had any persistent effects on the endogenous levels of the neurotransmitters DA NA and 5-HT or th r espectiv amino acid precursors tyrosine and tryptophan as measured in th whole brain at 28 days of age However the activity of the rat limiting enzymes tyrosine hydroxylase and tryptophan hydroxylase respectively was found to be impaired i the br ins of th animal exposed to a prolonged partial diminuation f neonatal oxygenation for 4 5 h during the neonatal period but not i the 4 weeks ld rats exposed to a brief episode of 20 min anoxi This difference suggests that the neonatal animal can manage shorter periods of asphyxia without subsequent neurochem ical damag However when the period f oxygen deprivation becomes more pro-

longed focal lesions in the brain may develop as a result of e g production of carbon dioxide and acidosis in areas with high metabolic activity (Windle 1966 Chen et al 1971)

Persistent effects on brain noradrenaline and serotonin metabolism have been found in rats exposed to asphyxia (45 min - 2 h) in infancy (Simon and Volicer 1976) In this study an increase in noradrenaline synthesis rate was found to persist after maturation We have at present no explanation to this discrepancy between these findings and our data but methodological differences e g production of asphyxia might contribute

Stress during pregnancy which causes a release of maternal catecholamines into the blood stream and a decrease in uterine blood flow thereby causing a brief period of anoxia has in animal experiments been found to produce persistent effects in catecholamine metabolism in discrete brain regions of the offspring after maturation The results from these types of studies are conflicting and increases (Huttunen 1971) as well as decreases (Moyer et al 1978) in noradrenaline turnover in discrete brain regions of the offspring have been described after maternal stress

Although conflicting results exist on the effects of eonatal oxygen deprivation on the monoamine metabolism at adult age it can be concluded that an asphyxic insult in the neonatal animal can produce permanent biochemical changes

3 5 Late effects of oxygen deprivation on conditioned avoidance response (IV)

Numerous studies performed on several anim l species have demonstrated behavioral effects after perinatal oxyge deprivation (see Meier 1971) These effects include both learning deficits and changes in emotionality

In parallel with the effects on monoamine synthesis discussed above we have found that a prolonged hypoxia (4 5 h 6% O_2) but not a brief period of anoxia (20 min) in the neonatal period interfered with the acquisition of a conditioned avoidance esponse in the 4 weeks old rats In view of the well-known importance of an undisturbed catecholamine system for the maintenance of conditioned avoidance behavior (see Engel and Carlsson 1977) it seems feasible to speculate that the persistent behavioral changes are closely carr lated to the biochemical impairment observed

In previous stud es from this laboratory Brown and coworkers have demonstrated that catecholamine pathways are invol ed in the disruption of conditioned avoidance

behavior and suppression of locomotor activity during acute hypoxia in the adult rat (Brown et al 1973 Brown and Engel 1973)

Henc catecholamines appear to be involved in the acute as well as late behavioral effects caused by exposure to hypoxia

3 6 Effects of monoamine receptor stim lating agents on survival during hypoxia (V)

Previous studies from this laboratory on adult rats (Brown et al 1975) have indicated that during hypoxia there is an inhibition of the physiological activity of the dopaminergic neurons i the br in esulting in a reduced nerve- mpulse induced release of DA while the nerve impulse induced release of NA during hypoxia was unaffected or possibly accelerated

Also in the neonatal anim l energy reserves are limited and the brain metabolic rat high (Jilek 1970) Hence neuronal acti ity must with necessity decrease when energy production declines during severe oxygen deprivation

When the precursor amino acids DOPA and/or 5-HTP were administered to th neonatal rats immediately befor 6% hypoxia thereby bypassing the oxygen-dependent rate-limiting steps a marked decrease in survival tim was observed Similar ly administration of a noradrenaline eceptor stim lating agent clonidine but not a dopamine receptor stimulating drug pomorphine decreased neonatal survival time during oxygen deprivation These results indicate that central catecholamine (principally noradrenergic) and indoleamine mechanisms may be involved in the factors determi ing survival during neonatal hypoxia It should be pointed out that NA pathways have been found to be r latively mature at birth whereas DA pathways develop at later stage during ontogeny (Kellogg and Lundborg 1973 Lundborg and Kellogg 1974)

It can be argued that the decreased survival tim of the neonatal rats during hypoxia may be due principally t peripheral mechanisms such as e g cardiovascular or pulmonary dysfunction However supporting our hypothesis of central mechanisms being involved are the ecent results by Berntman (1978) demonstrating that administration of catecholamines (adrenaline and noradrenaline) increases cerebral oxygen consumtion and induces changes i cerebral energy metabolism indicat ing that the low cerebral energy eserves may be a limiting factor If then stim lation of central adrenergic receptors decrease the survival tim it can be hypothetized that eceptor-blocking agents may increase survival in neonatal imals dur ing hypoxia Work are in progress to evaluate thi possibility

SUMMARY AND CONCLUSIONS

Based on the results of the present study it can be concluded that:

- hypoxia and anoxia in developing animals causes inhibition of tyrosine hydroxylase and tryptophan hydroxylase activity the first and rate-limiting enzymes of the catecholamine and indoleamine synthesis pathways, respectively
- the enzymes tyrosine hydroxylase and tryptophan hydroxylase were in general equally sensitive to hypoxia at the various ages studied In addition no regional differences were observed in the rat brai
- immediately after anoxia there is an increased availability of monoamine precursor amino acids and an increase i monoamine synthesis rate
- persistent biochemical and behavioral changes as measured as monoamine synthesis rate and conditioned avoidance response respecti ely occur after severe neonatal oxygen deprivation
- activation of the serotoninergic and noradrenergic neuronal systems decreases sur ival time during hypoxia in neonat l animals

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Studies on thiamine and neuromuscular transmission

By

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CONTENTS

The present survey is based on the following papers which will be referred to by their Roman numerals,

I *Radio-enzymatic assay of acetylcholine in tissues* Biochem Pharmacol 1975 24 1339-1341

II An improved method for the determination of endogenous thiamine and its phosphate esters in biological material Nutrition and Metabolism 1978 516 xxx

III *Evidence for the formation of a methylthiamine-like* substance in the rat phrenic nerve diaphragm preparation Biochem Pharmacol 1977 26 1321-1325

IV Release of thiamine and formation of a methylthiamine-like substance in the rat phrenic nerve-diaphragm preparation Acta physiol scand 1977 101 22-27

V Binding of thiamine to nicotinic receptors from Torpedo marmorata and the frog end plate Acta physiol scand 1978 103 154-159 (Together with L Elfman and B Rydqvist)

VI *Possible role of thiamine in neuromuscular transmission Accepted for publication in Europ J Pharmacol*

ABBREVIATIONS MTLS = methylthiamine-like substance PTP = post-tetanic potentiation and Th-DP = thiamine diphosphate

INTRODUCTION

Axonal transport of radioactive acetylcholine formed from radioactive choline injected directly into the tissue was studied in cat ventral roots (Waldenlind and Anderson 1978) In the chromatograms of extracts from such ventral roots an unidentified compound was found with chromatographical characteristics of methylthiamine A compound with the same chromatographical characteristics was also found in the chromatograms if radioactive thiamine had been injected into the tissue The compound was assumed to be methylthiamine but exact structural identification is lacking and the compound is therefore denoted as the methylthiamine-like substance (MTLS) The MTLS has been further characterized by chromatography its formation from different precursors has been studied and its formation in unstimulated and stimulated phrenic nerve diaphragms preparations has been compared Since it was thought that the formation of the MTLS might be related to a function of thiamine in neuromuscular transmission the effects of thiamine on neuromuscular transmission were also studied

The importance of thiamine for the nervous system was shown by Eijkman (1897) who demonstrated that degeneration of peripheral nerves occurred in fowls that were fed on thiamine-deficient rice About 40 years later Peters (1936) demonstrated that thiamine deficiency led to an increased level of lactic acid in the brain He showed that thiamine has a role in pyruvate metabolism It is now common knowledge that thiamine diphosphate (Th DP) serves as the coenzyme for pyruvate dehydrogenase α-ketoglutarate dehydrogenase and transketolase Several studies have shown

that the activities of these enzymes are reduced when there is a sufficient decrease in the levels of TH-DP in the tissues These biochemical changes are sometimes associated with clinical symptoms anorexia and weight loss cardiomegaly and bradycardia and neuromuscular disturbances (lameness ataxia and convulsion) together with other minor symptoms

After prolonged thiamine deficiency structural lesions of the central nervous system have been found in well-defined parts of the brain stem in various species (Prickett 1934 Evans et al 1942 Rinehart et al 1949 Dreyfus and Victor 1961) In the peripheral nervous system structural changes have been shown to occur in pigeons (Swank 1940 Shaw and Phillips 1945) whereas in other species (mice pigs rats and Rhesus monkeys) the results are contradictory (Wintrobe et al 1944 Dunn et al 1947 Rinehart et al 1949 North and Sinclair 1956) Since in the early stages of thiamine deficiency the symptoms can be reversed within hours by the administration of thiamine it has been thought that the morphological lesions are preceded by functional disturbance (Victor et al 1971)

Recent ultrastructural studies of the brain-stem lesions found in all species investigated including man suggest that the earliest changes produced by thiamine deficiency occur in the cell membrane A striking hypertrophy of the presynaptic boutons and a decrease in the number of synaptic vesicles were found in thiamine deficient rats by Tellez and Terry (1968) and by Pena and Felter (1973) It has also been shown that thiamine pyrophosphatase is localized in synaptic vesicles and in the terminal membrane in axon

terminals of the rat spinal cord (Griffith and Bondareff 1972 Knyihar et al 1973 Csillik et al 1974) indicating that this enzyme might be of importance for neurotransmission

After the biochemistry of Th DP dependent reactions had been clarified numerous attempts were made to relate the neurological symptoms following thiamine deficiency to a disturbance in these reactions Pyruvic acid metabolism transketolase function acetylcholine levels and γ amino-butyric acid formation have been investigated but the efforts to establish a correlation to the neurological symptoms have been unsuccessful (Gubler 1976) The biochemical mechanism behind the selective vulnerability of certain areas of the nervous system which is so characteristic of thiamine deficiency is still unexplained

Two thiamine antagonists oxythiamine and pyrithiamine have been used to produce experimental thiamine deficiencies In contrast to pyrithiamine oxythiamine has never been observed to induce the neurological symptoms that are regularly induced by pyrithiamine (Gubler 1976) It has been shown that oxythiamine enters the brain to only a slight extent whereas pyrithiamine is accumulated in the brain (Rindi and Perri 1961) This difference in distribution may explain why only pyrithiamine induces the neurological symptoms Pyrithiamine is a potent inhibitor of thiamine phosphokinase an enzyme which phosphorylates thiamine to Th DP (Koedam and Steyn Parvé 1960) Since thiamine is mainly stored as Th-DP in the body the storage capacity decreases in the presence of pyrithiamine and non phosphorylated thiamine is excreted in the urine (DeCaro et al 1958) Cooper and Pincus (1967) suggested that pyrithiamine might induce neurological symptoms by

a direct interaction with thiamine in the neural membrane and not by an interaction with thiamine requiring enzymes

Many authors have suggested an additional role for thiamine in the mechanism of nervous excitation In 1947 such a role was postulated by v Muralt The basis for his hypothesis was that if frog sciatic nerves were submitted to short tetanic stimulation then more thiamine could be extracted by a Ringer solution from the freeze-dried nerve powder than from a corresponding sample of unexcited nerves Since the daily requirements of thiamine are rather high v Muralt (1947) discussed the possibility that thiamine could be a metabolic substance and not only a catalyst How thiamine or thiamine compounds could participate directly in the metabolism of the nerve besides functioning as a catalyst could not be answered An unidentified thiamine metabolite (compound X) was described in extracts from frog sciatic nerves (v Muralt 1958) The amount of compound X increased when the nerves were submitted to tetanic stimulation and decreased in the resting period

It was shown by Kunz (1956) in v Muralt s laboratory that pyrithiamine blocked electrical activity at the node of Ranvier while oxythiamine had no effect Fern extract (containing thiamine destroying factors) had an action similar to that of pyrithiamine The blocking action of pyrithiamine was not reversed by thiamine The effects of pyrithiamine and fern extract were considered to be related to the Na-transport system necessary for the action potential Muralt s theory that thiamine may have a role in neuro conduction by activating the Na-transport system has however not been confirmed in experiments where the effect of

thiamine antagonists have been studied on voltage clamped squid giant axons by Goldberg and Cooper (1975) and Goldberg et al (1975) These authors considered the effects of the thiamine antagonists on nerve fibres to be due to a non specific stabilizing interaction with the nerve membrane and not to any antagonizing action on endogenous thiamine However Barchi (1976) who also used squid giant axons considered it still possible that the thiamine antagonists may interfere with the Na transport system necessary for the action potential

There are several other studies (fitting more or less together) where a possible neural function of thiamine has been investigated Thus Itokawa and Cooper (1969) found that thiamine and thiamine monophosphate were released from a perfused rat sciatic nerve or spinal cord preparation which had been exposed to low concentrations of acetylcholine tetrodotoxin ouabain and LSD Thiamine and thiamine monophosphate were released into the perfusion medium while 80 % of the thiamine-containing material in the nerves was in the form of Th DP and thiamine triphosphate Eichenbaum and Cooper (1971) showed that ultraviolet light of the wavelengths absorbed by thiamine abolished the action potentials in rabbit vagus nerve and that the addition of thiamine alone restored them Armett and Cooper (1965) found that the post-tetanic hyperpolarization in desheathed vagus nerve was abolished by pyrithiamine - an effect that was prevented by the addition of thiamine An impaired transmission in the superior cervical ganglion of the thiamine deficient rat has been shown to occur under high frequency stimulation whereas the conduction velocities and amplitudes of the action potentials in the sympathetic cord were unaffected (Perri et al 1970)

Indirect support according to Dreyfus (1976) for the view that the symptoms of thiamine deficiency are not the result of an impaired coenzyme function of Th DP is that the deficiency symptoms are much easier precipitated if the thiamine deficiency is combined with a large carbohydrate load The effect of the carbohydrate load was considered to be due to stimulation of the enzyme systems where Th DP is a coenzyme at the expense of another thiamine - requiring mechanism that is responsible for the deficiency symptoms

A possible function of thiamine in neuromuscular transmission was investigated in the present work by studying

1 the formation of the methylthiamine-like substance (MTLS) from choline and thiamine

2 the formation of the MTLS after nerve stimulation

3 the release of radioactive thiamine from the stimulated phrenic nerve-diaphragm preparation

4 the binding and effect of thiamine on nicotinic acetylcholine receptors and

5 the effect of thiamine on neuromuscular transmission in rat masseter muscle

COMMENTS ON METHODS

The different methods have been described in detail in the individual papers and therefore the methodological principles are only shortly summarized here

In paper I and II the methods for the extraction of the MTLS are given The MTLS was extracted from homogenized cat ventral roots using a counter ion dissolved in an organic solvent The homogenization was carried out in an organic solvent which has two advantages immediate denaturation of the proteins and a partial solubilization of the lipids These facts may explain why the MTLS was not found in earlier studies where choline metabolites have been extracted first after acid precipitation Accordingly when the ventral roots in the present study were homogenized in trichloroacetic acid no MTLS was recovered from the supernatant after centrifugation The occurrence of MTLS in the organic homogenization medium was considered improbable since only trace amounts of MTLS were found if radioactive line was added to nonradioactive ventral roots before the homogenization

In paper V the binding of thiamine to nicotinic acetylcholine receptors was studied both by using an isolated receptor protein from the electric organ of Torpedo marmorata and by using end plate receptors from frog sartorius muscle The isolated nicotinic acetylcholine receptor has certain characteristics in common with nicotinic receptors in the frog muscle end plate it binds nicotinic antagonists in low concentrations However the binding of acetylcholine to the isolated nicotinic acetylcholine receptor requires a high acetylcholine concentration (10^{6} M) whereas the

acetylcholine concentration that has an effect on the frog end-plate is much lower (10^{-9} M) Since differences in binding characteristics between the two types of receptors exist it is important to complete results achieved with the isolated receptor with results from frog muscle end plate receptors If the binding of a substance to nicotinic receptors is studied by using both techniques and the results are the same then the probability is increased that the binding of this substance to nicotinic acetylcholine receptors or to structures closely related to their function (ionophores) is studied

In paper VI the effect of thiamine on post-tetanic potentiation (PTP) was studied using the masseter muscle of the rat The masseteric nerve was stimulated with supramaximal impulses the voltage of the stimulation impulses being 150 % of that which yielded maximal muscle twitches Therefore the observed changes in muscle tension of the muscle contractions do not depend on differences in the recruitment of muscle fibres In some experiments field stimulation of the masseter muscle was made No difference in results between stimulation of the masseteric nerve and field stimulation was noted The pre tetanic and post-tetanic stimulation frequency was 0 2 Hz and the impulse duration 0 2 ms When the low frequency stimulation is interrupted by a short tetanus a transitory increase in the peak tension of the isometric muscle twitch response (PTP) is seen in fast mammalian muscle PTP in fast mammalian muscle has been described to be due to an increased contractility of the muscle fibres (Standaert 1964)

RESULTS AND DISCUSSION

Chromatographical characterization of the MTLS
When the radioactive ^{3}H-choline was injected into the ventral horn nuclei in the spinal cord of the cat radioactivity appeared in the ventral roots after less than 30 min (paper III) Part of the radioactivity was extracted by homogenizing the roots in allylcyanide containing sodium-tetraphenyloboron (counter ion extraction) Compounds which contain a quaternary ammonium ion as the single charge are thereby bound to the sodium-tetraphenyloboron and extracted into the organic phase (Fonnum 1969) By *adding a strong acid to the organic phase (e g HCl) the* quaternary ammonium compounds are regained into the inorganic phase which thereafter can be freeze-dried

When the freeze dried extracts from the radioactive ventral roots were dissolved in ethanol and submitted to paper chromatography then three peaks of radioactivity could be found Two peaks had as expected the chromatographical characteristics of acetylcholine and choline but the third one could not be identified as any known choline metabolite Thiamine was found to be the only substance with chromatographical properties similar to those of the unknown substance Since the methyl groups of the quaternary ammonium ion of choline were labelled the possibility was considered that the unknown compound was identical with methylthiamine formed by methylation

Methylthiamine was synthesized as described in paper III and chromatographed together with the unknown compound in

several chromatographical systems

In all chromatographical systems tried the unknown compound behaved as methylthiamine Radioactive thiamine was injected into the ventral horn nuclei Also in this case a radioactive peak could be found in the chromatograms of ventral root extracts Thus a compound (methylthiamine-like substance (MTLS) with the same chromatographical characteristics as methylthiamine may have been formed from both choline and thiamine The evidence for identity between the MTLS and methylthiamine is 1 chromatographical identity between the MTLS and methylthiamine in several chromatographical systems and 2 the MTLS is formed both from choline and thiamine Such evidence for identit between two compounds is only indirect and therefore the substance is called MTLS

MTLS and choline metabolism

The MTLS was formed in the ventral roots after the injection of radioactive choline It was considered to be of interest to study whether the MTLS was formed from choline itself or from any of its metabolites (paper III) Choline is metabolized in three directions 1 to acetylcholine 2 to phospholipids and 3 to betaine

Radioactive betain did not serve as a precursor for the MTLS when injected into the ventral horn nuclei Therefore it was considered improbable that the MTLS was formed after demethylation of betain or from any of its metabolites To study a possible relationship between the formation of the MTLS and phospholipids the time course for formation of the MTLS and phospholipids was compared The formation of

the MTLS was maximal after two hours Thus the formation of the MTLS does not seem to be dependent on the formation of phospholipids When the amounts of acetylcholine and MTLS in ventral and dorsal roots were compared the amounts of both acetylcholine and MTLS were higher in ventral roots than in the dorsal roots When the time course of the formation of acetylcholine and MTLS in ventral roots was studied it was found that both compounds reached their maximal level in about 30 min Thus it seems as if the formation of acetylcholine and of MTLS are related The immediate precursor for the MTLS can according to these studies be either choline or acetylcholine The biological importance *however of the formation of the MTLS was unclear The* idea was put forward that the formation of methylthiamine could reflect the activation of nicotinic receptors

The formation of the MTLS following administration of ^{3}H choline did not occur in liver kidney or spleen and may therefore be specific for nerve tissue and especially motor nerves Since it is not formed in all tissues it is improbable that the formation of the MTLS only represents a common break-down product of choline or thiamine

Formation of the MTLS in the stimulated rat hemidiaphragm To investigate whether the formation of the MTLS could be influenced by changes in nerve activity (as was the formation of compound X in v Muralt s study) the formation of the MTLS in the stimulated and nonstimulated phrenic nerve-dia phragm preparation was studied It was reported in paper IV that the amounts of MTLS formed were found to be higher in stimulated that in unstimulated rat diaphragms The formation of the MTLS was inhibited by d tubocurarine (12 mg/kg) The MTLS could only be extracted from the nerve

terminal region of the diaphragm muscle (can easily been seen as a band along the muscle) Thus the level of MTLS in the innervated part of the rat diaphragms increased as a result of nerve stimulation as did compound X in v Muralt s study (1958) In addition the formation of MTLS was inhibited by curare suggesting that the formation of the MTLS is linked to the activation of curare-sensitive receptors

Release of ^{35}S-thiamine from the stimulated rat diaphragm When the phrenic nerve of the hemidiaphragms was stimulated a release of radioactive thiamine into the bath was found in the presence of d-tubocurarine (paper IV) Since the muscle contractions were blocked by d tubocurarine the thiamine in the bath after nerve stimulation probably originated from the nerve fibres Thiamine is mainly stored as phosphates Therefore a dephosphorylation of thiamine or an increase in extractable non phosphorylated thiamine seems to occur during nerve stimulation In electrically stimulated frog sciatic nerves v Muralt (1947) found an increase in thiamine extracted by Ringer solutions results which showed that the amount of free (=extractable) thiamine had increased as a result of the stimulation A dephosphorylation of thiamine phosphates was noted by Itokawa and Cooper (1970) who found an increase in non phosphorylated thiamine in the perfusion fluid from rat sciatic nerve or rat spinal cord when neuroactive agents such as acetylcholine tetrodotoxin ouabain or LSD were added to the perfusion fluid Thus electrical stimulation of or addition of certain neuroactive agents to nervous tissue might induce a shift from bound to free thiamine and /or/ a dephosphorylation of the thiamine phosphates

Binding of thiamine to nicotinic acetylcholine receptors

Since the increase in the MTLS in the phrenic nerve-diaphragm preparation was blocked by d-tubocurarine it was considered to be of interest to study a possible binding to and effect of thiamine on nicotinic acetylcholine receptors Nicotinic acetylcholine receptors can be isolated and purified from electric organs of fish (Eldefrawi and Eldefrawi 1973 Heilbronn et al 1974 Klett et al 1973 Meunier et al 1974) Such material was considered to be suitable for binding studies In paper V it was shown that thiamine binds reversibly to the isolated nicotinic acetylcholine receptor protein (K_D=10 ^{5} M) The binding of thiamine was inhibited by *the venom toxin from Naja naja siamensis which is a specific* antagonist for postsynaptic nicotinic receptors (Chang and Lee 1966) The binding of thiamine to the isolated nicotinic receptor was not inhibited by acetylcholine

A possible effect of thiamine on post-synaptic nicotinic acetylcholine receptors was investigated by studying the miniature end plate potentials of the frog muscle end plate (paper V) The miniature end plate potentials are the result of nicotinic acetylcholine receptor activation due to release of acetylcholine When thiamine (10 ^{5} M) was applied to the end plate preparation the amplitude of the miniature end plate potentials decreased reversibly and significantly No effect on the resting membrane potential was noted The decrease in the miniature end plate potentials induced by thiamine indicated that thiamine blocks the action of acetylcholine A decrease in the miniature end plate potentials by thiamine can be induced by a block of the sodium channels in the end plate that give rise to the miniature end plate potentials registered Since the binding of acetylcholine

was not inhibited by thiamine in the isolated receptor from <u>Torpedo</u> <u>marmorata</u> these results could indicate that thiamine binds to the sodium channels and not to the acetylcholine binding site

Pyrithiamine and neuromuscular transmission

In paper VI the effect of thiamine on neurotransmission at higher stimulation frequencies (> 1Hz) was studied in rat masseter muscle It is a typical fast muscle since it responds with individual twitches up to frequencies of 30 Hz Since the masseter muscle is fast the tension of the individual muscle twitches will increase at high stimulation frequencies (stair case effect) and a frequency-dependent increase in contractility (PTP) will develop after moderate tetanic stimulations

The effect of thiamine on the development of PTP in the untreated masseter muscle was investigated by administering thiamine intravenously No effect was noted on the PTP or on the ordinary single twitches When the effect of pyrithiamine and thiaminase-containing preparations (fern extract on the PTP was studied then the muscle twitches at stimulation frequencies of > 1 Hz were decreased and PTP was abolished Intravenous injections of thiamine alone there after restored the PTP and the muscle twitches at high frequency stimulation Oxythiamine had no effect on the muscle twitches induced by high frequency stimulation or on the PTP No effect on directly excited muscle twitches was seen after pyrithiamine administration Therefore the effects of pyrithianine and thiamine are on the nerve and not on the muscle

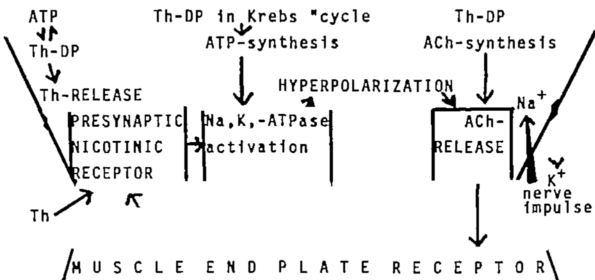
Pyrithiamine administration caused a concommittant decrease in the PTP and the muscle twitches at stimulation frequencies of > 1 Hz The effect of pyrithiamine on neurotransmission at high stimulation frequencies is due to an impaired neuromuscular transmission that is necessary for the development of the frequency-dependent increase in contractility (staircase and PTP) since the directly-stimulated muscle twitches at high frequency stimulation were unaffected

The impairement of neurotransmission induced by pyrithiamine that was reversed by thiamine could be due to a decreased excitability of the presynaptic nerve fibers or /and/ of the end-plate membrane Metabolic effects such as a decreased synthesis of acetylcholine or ATP or impairement of a specific function of thiamine in the nerve membrane could be the cause for the decrease in excitability

Possible mechanisms of action of thiamine

A possible function of thiamine in neurotransmission is its role as a coenzyme for pyruvate dehydrogenase an enzyme which regenerates acetyl groups and which therefore is of importance for acetylcholine synthesis and for the regeneration of ATP in the citric acid cycle However experimental evidence does not support the view that such a mechanism of action of thiamine is of importance for the symptoms in thiamine-deficient animals In thiamine deficiency the acetylcholine levels are described to be either elevated or decreased (Gubler 1976) and the ATP-levels normal or elevated (Holowick _et al_ 1968 McCandless and Schenker 1968 Inoue _et al_ 1970) In the present study d-tubocurarine which blocks the effect of acetylcholine, induced a similar decrease in the PTP and reversal of the stair case

effect as did pyrithiamine This result however could be consistent with the view that pyrithiamine inhibits a function of acetylcholine in the masseter muscle preparation that is necessary for the development of PTP and maintenance of muscle twitches at stimulations above 1 Hz If thiamine deficiency or pyrithiamine administration do not change the levels of acetylcholine or the energy supply (ATP production) another possibility could be that thiamine is necessary for the change in nervous excitability induced by acetylcholine Pyrithiamine has been shown to inhibit the post tetanic hyperpolarization (Cooper and Pincus 1967) without affecting the Na K-ATPase results which suggest that thiamine is of importance for maintenance of the membrane potential at high stimulation frequencies Acetylcholine has been shown to increase the hyperpolarization in C-fibers of rabbit vagus nerve (Armett and Ritchie 1960) This effect of acetylcholine was inhibited by d-tubocurarine and hexamethonium but not by atropin in corresponding doses Thus both acetylcholine and thiamine might be of importance for maintaining the membrane potential at high frequency stimulation The efficiency of neurotransmission is directly dependent on the degree of membrane polarization (Eccles 1964) Therefore it is possible that acetylcholine and pyrithiamine also are able to affect the release of transmitter In accordance with this view are the results where acetylcholine has been shown to be important for maintaining the neurotransmission at higher stimulation frequencies via a presynaptic action (Bowman and Webb 1973 Beranek and Vyskocil 1967 Hubbard et al 1969) Thiamine could as already mentioned be of importance for the synthesis of acetylcholine and /or/ ATP and thus influencing the effects of acetylcholine on the membrane potential If however



(ACh=acetylcholine Th=thiamine and Th-DP= thiamine diphosphate)

Fig 1 Thiamine and neuromuscular transmission
Possible sites of action for thiamine
A schematic picture of the presynaptic nerve terminal and the muscle end plate is shown Possible mechanisms of action for thiamine in maintaining the neuromuscular transmission are illustrated The different mechanisms of action for thiamine are described in the text under results and discussion

pyrithiamine blocks a function of thiamine which is unrelated to the thiamine-requiring enzymes then it is possible that thiamine is of importance for the excitability of presynaptic structures (nicotinic receptors) activated by acetylcholine that are of importance for maintaining neurotransmission at high stimulation frequencies

In paper III the hypothesis was put forward that the formation of the MTLS may reflect the activation of nicotinic receptors Evidence for such a hypothesis should include 1 decreased formation of the MTLS in the presence of d tubocurarine 2 a binding of thiamine to nicotinic receptors and 3 an effect of thiamine on the function of nicotinic receptors A decreased formation of the MTLS after administration of d tubocurarine was shown in paper IV and a specific binding to nicotinic receptors in paper V However addition of thiamine to nicotinic receptors of the frog end plate decreased the miniature end plate potentials Thiamine was also specifically bound to an isolated nicotinic receptor protein from Torpedo marmorata a binding which was inhibited by a venom toxin with specific affinity to nicotinic receptors but not by acetylcholine Thus these results suggest that thiamine was bound to the sodium channels necessary for the miniature end plate potentials The specific binding of thiamine to the acetylcholine-activated sodium channels might imply that thiamine is important for the activation of these channles in lower concentrations Unfortunately this possibility can not be supported or disclosed by the experiments in paper V since an experimental thiamine deficiency was not induced

Any direct evidence showing that formation of the MTLS reflects activation of nicotinic receptors was not obtained in the present study The experiments performed on rat masseter muscle shows that the effects of pyrithiamine and d-tubocurarine on the stair-case effect and the PTP are similar and are possible to explain if it is assumed that thiamine is of importance for a presynaptic action of acetylcholine necessary for the membrane potential

In paper IV an increase in nonphosphorylated thiamine was found in the stimulated phrenic nerve diaphragm preparation Similar results were described by v Muralt (1947) from stimulated frog nerve Thus electrical stimulation of nerve tissue seem to increase the amount of nonphosphorylated thiamine suggesting an increased turn over of thiamine at electrical stimulation These results can be in accordance with the view that thiamine compounds are of importance for miantaining the membrane potential presumably by influencing the ion transport through the membrane since electrical stimulation will activate the mechanisms that are necessary for maintaining the membrane potential

An increase in an unidentified thiamine compound (compound X) after nerve stimulation has been described by v Muralt (1958) An increase in a thiamine metabolite (MTLS) after nerve stimulation is described also in this study These results also indicate that there is an increase in the turnover of thiamine at nerve stimulation

In the present work it was found that the increase in the MTLS seen after nerve stimulation was inhibited by d tubo curarine A frequency dependent decrease in the excitability

of the nerve terminal fibres is seen after d tubocurarine administration (see paper VI for references) Thus these results are in accordance with the view that there is a turn-over of thiamine compounds in nerve tissue that is related to membrane excitability

The present results support the hypothesis that thiamine is of importance for membrane excitability especially at neuromuscular synapses and that there is a turn-over of thiamine compounds In the present study it has been shown that the neuromuscular transmission is impaired at an early stage by the thiamine antagonist pyrithiamine If pyrithiamine is antagonizing a function of thiamine necessary for membrane excitability then the neuromuscular transmission apparently is the most vulnerable localization for a dysfunction of the peripheral motor system due to pyrithiamine administration

The importance of a synaptic dysfunction also in thiamine deficiency whatever the biochemial mechanism behind the dysfunction may be is supported by the morphological studies carried out on brains from thiamine deficient rats A hypertrophy of the synaptic boutons and a decrease in the number of synaptic vesicles were shown in thiamine-deficient rats (Tellez and Terry 1968 Pena and Felter 1973) In addition the importance of synaptic failure in thiamine deficiency may also be supported by the fact that the symptoms in the early stages of thiamine deficiency can be reversed within a few hours (Dreyfus 1976) Thiamine reaches the axon membranes via axoplasmic transport (Tanaka _et al_ 1976) and it is improbable that thiamine which is a quaternary amine could reach the axon membrane by passive diffusion Since

the deficiency symptoms are reversed within a few hours and the axoplasmic transport of thiamine to the entire axons probably will take place in a much longer time, the most probable site of membrane dysfunction in the early stages of thiamine deficiency is in the terminal boutons which are unprotected by myelin and thus accessible to the administration of thiamine

CONCLUSIONS

1 The formation of a methylthiamine-like substance (MTLS) in nerve tissue is described

2 The formation of the MTLS reached its maximum at the same time as the formation of acetylcholine but significantly earlier than the formation of phospholipids No MTLS was formed from betain

3 The formation of the MTLS increased with nerve stimulation and was inhibited by d-tubocurarine The role of the formatior of the MTLS remains unknown However it is an interesting possibility that the formation of the MTLS is an indicator of a specific role of thiamine for membrane excitability

4 Thiamine is released from the curarized phrenic nerve-diaphragm when the phrenic nerve is stimulated

5 Thiamine binds to isolated nicotinic acetylcholine receptors from Torpedo marmorata and to nicotinic acetylcholine receptors of the frog end plate

6 Pyrithiamine and fern extract thiamine antagonists impaires neurotransmission in rat masseter muscle via a frequency dependent mechanism The effect of pyrithiamine and fern extract is reversed by thiamine Oxythiamine has no effect on the neurotransmission in rat masseter muscle

7 The present results are consistent with the view that thiamine is important for the excitability of the nerve terminals by an effect on the active ion transport that is necessary for regeneration and maintenance of the membrane potential

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PROSTAGLANDINS AND KIDNEY FUNCTION

An Experimental Study in the Rabbit

By

ERNST OLIW

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I *In vivo* inhibition of prostaglandin synthesis in rabbit kidney by nonsteroidal anti inflammatory drugs *Acta pharmacol* (Kbh) 1978 *42* 179 184
(Together with I LUNDEN and E ÄNGGARD)

II Indomethacin and diclofenac sodium increase sodium and water excretion after extracellular volume expansion in the rabbit *Europ J Pharmacol* 1978 *49* 381 388
(Together with G KÖVER C LARSSON and E ÄNGGARD)

III Different effects of furosemide on urinary excretion of prostaglandin E_2 and $F_{2\alpha}$ in rabbits *Acta physiol scand.* 1979 in press
(Together with E ÄNGGARD)

IV The influence of dietary sodium on urinary prostaglandin excretion *Acta physiol scand.* 1978 *103* 100 106
(Together with D DAVILA T DAVILA and E ÄNGGARD)

V Determination of 6 keto prostaglandin $F_{1\alpha}$ in rabbit kidney and urine and its relation to sodium balance *Acta physiol scand* 1979 in press
(Together with I LUNDEN B SJÖQUIST and E ÄNGGARD)

VI Acute unilateral ureteral occlusion increases plasma renin activity and contralateral urinary prostaglandin excretion in rabbits *Europ J Pharmacol* 1978 *53* 95 102

CONTENTS

A B B R E V I A T I O N S

Th following common names and abbreviations have been used:

Arachidonic acid: all-cis-5 8 11 14-e cosa tetraenoic acid
ADH: antidiuretic hormone
GC-MS: gas chromatography- mass spectrometry
PAH paraaminohippuric acid
PRA plasma renin activity
Prostacy lin (PGI_2): see prostaglandin I_2
Prostaglandin A_2 (PGA_2) 15S-hydroxy-9-ketoprosta-5 10 13-trienoic acid
Prostaglandin B_2 (PGB_2): 15S-hydroxy-9-ketoprosta-5 8 (12) 13-trienoic acid
Prostaglandin D_2 (PGD_2): 9α 15S-dihydroxy-11-ket prosta-5 13 -di noic acid
Pro taglandin E_2 (PGE_2) 11α 15S-dihydroxy-9-ketoprosta-5 13 -dieno c acid
6-keto-prostaglandin $F_{1\alpha}$(6-keto-$PGF_{1\alpha}$): 9α 11α 15S-trihydroxy-6-ketoprost -13-enoic cid
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$): 9α 11α 15S trihydroxypro ta-5 13-dienoi acid
Prostaglandin $F_{2\beta}$ ($PGF_{2\beta}$): 9β 11α 15S-trihydr xyprosta-5 13 -dienoic acid
Prostaglandin G_2 (PGG_2): 15S-hydrop roxy-9α 11α-pe oxidoprosta-5 13--dienoic a id
Prostaglandin H_2 (PGH_2) 15S-hydroxy-9α 11α-peroxidoprosta-15 13--dienoic acid
Pro taglandin I_2 (PGI_2) 6 9α-epoxy-11α 15S-dihydroxyp o ta-5 13--dienoi acid
Sar lasin (Sa [1] Ala [8]) angioten in II
Thromboxan A_2 (TXA_2) 9α 11α-oxy-15S-hydroxythromba-5 13-di noic acid
Thromboxan B_2 (TXB_2) : 9α 11α 15S-trihydroxythromba 5 13-di noic acid
TLC Thin layer chromatogr phy

INTRODUCTION

Die hier beschriebene äther- und wasserlösliche Substanz mit blutdrucksenkender Wirkung sowie erregender Wirkung an verschiedenen isolierten glattmuskeligen Organen wird vorläufig „Prostaglandin" genannt
(U S von Euler 1935)

Prostaglandins constitute a family of acidic lipids with a wide spect rum of effects In the kidney prostaglandins may modulate blood flow and the release and effects of other renal hormones The present survey will also discuss prostaglandins in relation to renal handling of so dium and effects of diuretic drugs

Biosynthesis of prostaglandins

Prostaglandins are formed from eicosapolyenoic acids with 3 to 5 double bonds 8 11 14-eicosatrienoic acid 5 8 11 14-eicosatetraenoic acid (arachidonic acid) and 5 8 11 14 17-eicosapentaenoic acid (cf Bergström et al 1964 van Dorp et al 1964) Of these arachidonic acid is the most abundant in animal tissues Arachidonic acid is stored esterified to phospholipids in cell membranes After cleavage of the ester bond by phospholipases or by other means the free arachidonic acid can be metabolized by lipoxygenases and by prosta glandin endoperoxide synthase (Fig 1)

Lipoxygenases oxidize arachidonic acid to hydroperoxy and hydroxy eicosatetraenoic acids In human platelets 12 hydroperoxy and 12 hydroxy-eicosatetraenoic acids are formed and in rabbit leucocytes 5 hydroxy eicosatetraenoic acid (Hamberg and Samuelsson 1974 Nugteren 1975 Borgeat et al 1976) These reactions have as yet not been demonstrated to occur in the kidney Their importance under *in vivo* conditions is also unknown

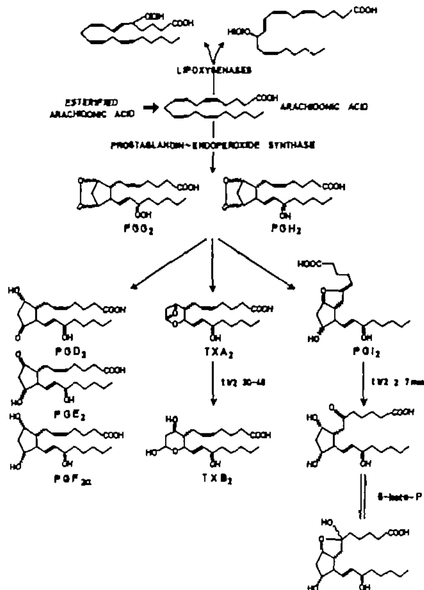


Fig 1 Some out in the metabolism of ar hidoni acid Arachidoni acid can be metabolized by *lipoxygenases* to hydroperoxy- and hydroxy-eicosatetraenoic acids or by *prostaglandin (PG) endoperoxide synthase* to the prostaglandin and p roxides PGG_2 and PGH Prostaglandin ndoper xid ar unstable in water solutions with a half lif of 5 min at 37°C *Prostaglandins* e g PGD_2 PGE_2 and $PGF_{2\alpha}$ can be formed non-enzymatically from the p ostaglandin ndoperoxid s but enzymatic isomerization t PGD_2 and PGE_2 and red ction to $PGF_{2\alpha}$ have been

The prostaglandin endoperoxide synthase (EC 1 14 99 1) converts arachidonic acid into the prostaglandin endoperoxide PGG_2 through a cyclo-oxygenase reaction followed by a peroxidase reaction to yield the prostaglandin endoperoxide PGH_2 (Miyamoto et al 1976 Hemler et al 1976 van der Ouderaa et al 1977 Bhat et al 1978) Experimental evidence for the existence of prostaglandin endoperoxides as intermediates in prostaglandin biosynthesis was obtained already in 1965 by Sameulsson Isolation of prostaglandin endoperoxides was accomplished in 1973 (Hamberg and Samuelsson Nugteren and Hazelhof) The isolated prostaglandin endoperoxides could readily be converted into prostaglandins

Studies on the metabolism of PGG_2 and PGH_2 led to the discovery of two novel compounds of considerable biological interest called thromboxanes and prostacyclin respectively (Hamberg et al 1975 Moncada et al 1976) The metabolism of arachidonic acid to prostaglandins and related substances is described in Fig 1 (cf Samuelsson et al 1978a for review)

The metabolism of arachidonic acid in various tissues to prostaglandins and related substances depends on the availability of prostaglandin endoperoxide synthase cofactors and the prostaglandin endo-

found in some tissue (Christ-Hazelhof t al 1976 Ogino et al 1977 Abdel Halim et al 1977) The enzyme omplex which f rms pr staglandins from their pr curso acids r often ref rred to as prostaglandin synthase Prostaglandin synthase has been found in almost all animal tissue b t pa ti ularly in the p eminal v sicl s and in rabbit enal medulla (Ch ist and van Dorp 1972) *Thromboxane synthase* wa discovered in human plat l t by Hamberg et al (1975) who proposed the hemical tructure of thromboxane (TXA_2) and it metabolit TXB_2 TXA_2 is unstable i wat solutions ($t_{\frac{1}{2}}$ 30-40 sec) and forms TXB_2 with los f bi logical activity TXA_2 is of physiological importance f r the plat l t aggregation re tion (Samuel son t al 1978b) *Prostacyclin synthase* was discove ed in art rial wall by Moncada t 1 (1976) PGI_2 is unstabl in wat r solution ($t_{\frac{1}{2}}$ 3-7 min) and hydrolyses to 6-k to-$PGF_{1\alpha}$ with lo s of biological activity (Cho and Allen 1978 Armstrong et al 1978)

peroxide metabolizing enzymes The latter enzymes seem to be rather unevenly distributed in the tissues It is expected therefore that each tissue will be capable of generating a profile of prosta glandin endoperoxide metabolites characteristic of that tissue Thus thromboxanes are found in platelets and PGI_2 in vascular walls (Pace Asciak 1977b Samuelsson et al 1978a Vane 1978)

Prostaglandin endoperoxides prostaglandins and thromboxanes appear to act locally rather than to be hormones by the classical definition of blood borne messengers The prostaglandin endoperoxides and TXA_2 are chemically unstable Many prostaglandins are also inactivated by metabolism during passage through the lungs (eg Änggård and Samuels son 1966 Piper et al 1970) PGI_2 on the other hand is only partly metabolized by the lungs and might be a hormone in the classical sense (Moncada et al 1978 Waldman et al 1978 Gerkens et al 1978)

Renal biosynthesis of prostaglandins

In 1965 Lee and his collegues described a vasoactive compound in rab bit renal medulla Later work revealed that it was a mixture of PGE_2 and PGA_2 (see Lee et al 1976 for review) Hamberg (1969) demonstra ted the synthesis of PGE_2 from exogenous arachidonic acid in homo genates of rabbit renal medulla Cortical biosynthesis of PGE_2 and $PGF_{2\alpha}$ was demonstrated more recently (Larsson and Änggård 1973 1976)

The *in vitro* biosynthesis of prostaglandins in the rabbit renal medulla has now been well characterized PGE_2 $PGF_{2\alpha}$ and PGD_2 are quantitatively most important (Blackwell et al 1975 Schwartzman et al 1976 Friesinger et al 1978) Prostaglandins are not stored in the kidney (Änggård et al 1972) The rate limiting step in prosta glandin biosynthesis is generally considered to depend on the amount of free arachidonic acid available (Samuelsson 1970) The release of free arachidonic acid is influenced by phospholipase A_2 which would appear to be the regulatory enzyme (Flower and Blackwell 1976) Brady kinin angiotensin II and ADH have been proposed to stimulate phospho

lipase A_2 activity leading to release of arachidonic acid and an increased prostaglandin synthesis *in vitro* (Kalisker and Dyer 1972 Danon et al 1975 Zusman and Keiser 1977)

The localization of prostaglandin endoperoxide synthase in the kidney has been studied by histochemical (Janszen and Nugteren 1971) and immunochemical methods (Smith and Wilkin 1977) The enzyme has been found particularly in the collecting ducts The occurrence of prosta cyclin and thromboxane synthase in the kidney has not yet been in vestigated

PGI_2 is converted to 6 keto $PGF_{1\alpha}$ by hydrolysis (cf Fig 1) Renal cortical microsomes have been found to form 6 keto $PGF_{1\alpha}$ from arachi donic acid and from prostaglandin endoperoxides *in vitro* (Zenser et al 1977 Whorton et al 1978) However the intrarenal distribu tion of prostacyclin synthase is unknown

Renal synthesis of TXA_2 has been established in the hydronephrotic kidney of the rabbit on challenge with bradykinin (Morrison et al 1977) Rat renal microsomes can form TXB_2 (Zenser et al 1977) but the amount of thromboxanes in the kidney is normally low and pre sumably without significance (Pace Asciak 1977b Morrison et al 1977)

The local action of prostaglandins in the kidney is terminated by removal of the prostaglandins to venous blood and to urine or by catabolism Renal cortex contains 15-hydroxyprostaglandin dehydro genase type I which inactivates prostaglandins (Änggård et al 1971 Larsson and Änggård 1973 Pace Asciak et al 1977) Renal cortex and medulla also contain enzymes which dehydrogenate both 9 and 15 hy droxyprostaglandins (Katzen et al 1975 Stone and Hart 1975) These enzymes have not yet been separated (cf Lin and Jaraback 1978) and are referred to as type II 15-hydroxyprostaglandin dehydrogenase and 9-keto reductase respectively (Lee and Levine 1975 Stone and Hart 1975) The latter might be important for interconversion of prosta glandins E to F (Weber et al 1977b)

Functional studies on renal prostaglandins

Studies on prostaglandins in the kidney have often aimed at examining (1) the effects of exogenous prostaglandins and related substances or (2) the effects of an increased or a reduced prostaglandin synthesis or

(3) the renal prostaglandin synthesis under variations in renal function (for review see Dunn and Hood 1977 Andersson et al 1976 or Zins 1976)

Many observations on the *in vitro* effects of exogenous prostaglandins have been of interest Grantham and Orloff (1968) demonstrated that PGE_1 counteracts the effects of ADH on water permeability in collecting ducts Other studies showed that arachidonic acid and PGI_2 stimulated renin release from slices of rabbit renal cortex (Weber et al 1976 Whorton et al 1977) These findings indicate that renal prostaglandins could modify the release and effects of other renal hormones

Many studies have shown that prostaglandins might effect renal reabsorption of sodium However following *in vitro* applications of prostaglandins both stimulatory and inhibitory effects have been found (Fülgraff and Brandenbusch 1974 Fine and Trizna 1977 Dunn and Howe 1977 Kauker 1977 Iino and Imai 1978) *In vivo* arterial infusion of PGE_2 PGD_2 or PGI_2 may increase renal blood flow sodium excretion and renin release (Gerber et al 1978a Friesinger et al 1978) The physiological significance of the renal effects of exogenous prostaglandins can be questioned The level of prostaglandins in the renal artery is normally lower than during infusion experiments Furthermore, infusion of one selected prostaglandin is not likely to mimic the effects of prostaglandin endoperoxides and their metabolites in the kidney

A more physiologically relevant way to study the effects of renal prostaglandin synthesis on renal function might be through the ad

ministration of arachidonic acid by using the precursor overload technique Arachidonic acid increases renal blood flow renin release and sodium excretion and these effects can be blocked by inhibitors of prostaglandin synthesis (Larsson et al 1974 Tannenbaum et al 1975 Bolger et al 1976 Gerber et al 1978b)

The effects of a reduced prostaglandin biosynthesis can be studied by depriving the experimental animal of prostaglandin precursor acids (essential fatty acids van Dorp 1976) or by blocking prostaglandin biosynthesis The latter method has been widely used since 1971 after Vane and coworkers had made the important discovery that aspirin indomethacin and other nonsteroidal anti inflammatory drugs inhibit prostaglandin biosynthesis (see Vane 1978 for review) These drugs block the cyclooxygenase reaction The formation of prostaglandin endoperoxides and their metabolites are therefore inhibited to the same extent

The nonsteroidal anti inflammatory drugs have been found to reduce renal blood flow especially in response to vasoconstrictor stimuli (Zins 1975) These drugs also block renin release *in vivo* and *in vitro* (see Dunn and Hood 1977 for review) and augment the renal effects of ADH (Andersson et al 1976) However renal sodium reab sorption is variably affected these drugs can reduce sodium excretion in anesthetized dogs (eg Feigen et al 1976) or increase it in unanesthetized animals (Kirschenbaum and Stein 1976)

Changes in renal prostaglandin synthesis *in vivo* have been estimated from the secretion of prostaglandins to renal blood or from the excretion of prostaglandins in urine The former method may over estimate the renal prostaglandin secretion due to contributions of prostaglandins formed by the blood during blood sampling (Samuelsson et al 1975) Urinary excretion of PGE_2 and $PGF_{2\alpha}$ is probably a better indicator of renal prostaglandin biosynthesis (Dunn and Hood 1977) Renal prostaglandin biosynthesis and urinary excretion of

PGE_2 and $PGF_{2\alpha}$ change in parallel (Frölich et al 1975 Dunn et al 1978) This assumption is also based on the low arterial levels of PGE_2 and $PGF_{2\alpha}$ (Samuelsson et al 1975) and on the metabolism of extrarenal prostaglandins during circulation through the kidneys and their excretion in urine as C_{16} or C_{18} metabolites (Granström 1972) PGE_2 and $PGF_{2\alpha}$ in urine are therefore likely to originate from the kidneys or the urinary tract

Previous studies have indicated that renal prostaglandins might be of importance in modulation of renal blood flow for the renal effects of ADH and for renin relea e The renin angiotensin system is gene rally believed to be involved in sodium retaining mechanisms in the kidney (Davis and Freeman 1976) The role of the renal prostaglandins in the local regulation of sodium excretion is unsettled The present studies focus on the role of prostaglandin in the control of sodium excretion in the rabbit kidney specifically in relation to sodium balance to activation and action of the renin angiotensin system and to the effects of loop diuretics such as furosemide

The results were achieved by a combination of pharmacological and biochemical techniques Nonsteroidal anti inflammatory drugs were used to study the renal effects of a reduced prostaglandin bio synthesis Chemical and radioimmunological methods were used to measure prostaglandins in kidney and in urine After characteriza tion of these techniques the aims were to study the renal prosta glandins and renal function

- (i) During acute changes in extracellular fluid volume
- (ii) During chronic changes in sodium intake
- (iii) In relation to the renal effects of furosemide
- (iv) In relation to the renin angiotensin sy tem

METHODS

The principles of the methods and of the experimental procedures will be surveyed Some complementary remarks are added in addi tion to the descriptions in papers I VI

Mass fragmentographic analysis of PGE_2 $PGF_{2\alpha}$ and 6 keto-$PGF_{1\alpha}$ (I, IV V)

Prostaglandins were measured by mass fragmentography In this technique (Axén et al 1971) a deuterated internal standard is added to the biological sample prior to purification procedures In the final mass spectrometric analysis the endogenous amount in the sample can then be obtained from the deuterium protium ratio of the sample

Determination of PGE_2 and $PGF_{2\alpha}$ The renal concentration of PGE_2 and $PGF_{2\alpha}$ increases rapidly after death (Änggård et al 1972) The time for the accumulation of prostaglandins was therefore set to 5 minutes (I V) Prostaglandins in the renal tissue samp les were purified by established methods *via* homogenization centrifugation chromatography on Amberlite XAD-2 (Gréen 1971) and separation of PGE and PGF compounds by silicic acid chromato graphy using step wise elution (Samuelsson 1963)

Many derivatives of $PGF_{2\alpha}$ suitable for analysis by gas chromato graphy-mass spectrometry (GC MS) have been described (eg Axén et al 1971 Sugiura and Hirano 1974 Middleditch and Desiderio 1973a) *The methyl ester trimethylsilyl ether* derivative of $PGF_{2\alpha}$ was chosen for analysis because of favourable mass frag mentographic properties (eg Oswald et al 1974) The ketogroup of E prostaglandins has to be derivatized prior to GC MS ana lysis eg by methoxylation or by dehydration of PGE to PGB com pounds (Gréen 1969 Middleditch and Desiderio 1973b Nicosia and

Galli 1974) Since analysis of $PGF_{2\alpha}$ was relatively easy PGE_2 was reduced to a mixture of $PGF_{2\alpha}$ and $PGF_{2\beta}$ by sodium borohydri de and derivatized to the methyl ester trimethylsilyl ether of $PGF_{2\alpha}$ and $PGF_{2\beta}$

Determination of 6-keto-$PGF_{1\alpha}$ The mass fragmentographic analy sis was performed essentially as described by Pace Asciak (1977a The urine and kidney samples were purified by preparative thin layer chromatography (TLC) The analysis was associated with some initial difficulties since unlike other prostaglandins 6 keto $PGF_{1\alpha}$ could not be stored in absolute ethanol and 6 keto $PGF_{1\alpha}$ and its lactol forms separate in some TLC systems

Comments on mass fragmentography Mass fragmentography is charac terized by high precision specificity and sen itivity if the deuterated standard contains little of the protium form and if the biological samples contain little interfering material in the final mass fragmentographic analysis To exclude such non spe cific interference it was essential to demonstrate that dif ferent fragments of the prostaglandin derivatives gave the same deuterium protium ratio (I V)

PGE_2 was measured after reduction to $PGF_{2\alpha}$ and $PGF_{2\beta}$ This step reduced the specificity since other substances could also be converted to PGF_2 However this problem was minimized by puri fying PGE_2 in the samples before reduction and by using $PGF_{2\beta}$ which is not naturally occurring in the final analysis (see I for discussion)

Radioimmunoassays for $PGF_{2\alpha}$ and PGE_2 (I VI)

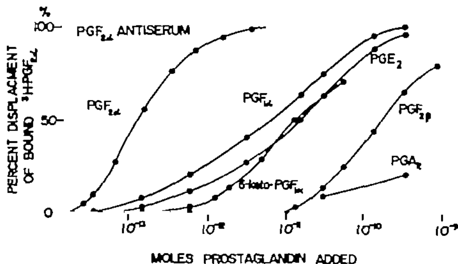
Mass fragmentographic determination of prostaglandins was not possible in series of small urine samples obtained during acute experiments Radioimmunoassays for PGE_2 and $PGF_{2\alpha}$ were there fore developed because of their potential high capacity and sen

sitivity The radioimmunoassay methods for analysis of prosta glandins were recently reviewed by Granström (1978)

Antisera against $PGF_{2\alpha}$ and $PGF_{2\beta}$ Antibodies against $PGF_{2\alpha}$ were raised in rabbits by immunization with $PGF_{2\alpha}$ coupled to bovine serum albumin Water soluble carbodiimide was used in the coupling reaction (Caldwell et al 1972) PGE_2 is unstable under these conditions (Levine 1973) However PGE_2 in biological samples can be quantitatively reduced to a mixture of $PGF_{2\alpha}$ (30-40%) and $PGF_{2\beta}$ (60 70%) by treatment with sodium borohydride (Lindgren et al 1974) The $PGF_{2\alpha}$ and $PGF_{2\beta}$ formed can both be mea sured by radioimmunoassay (Lindgren et al 1974) This method was adopted and antibodies were raised against $PGF_{2\beta}$ in the same way as described above for $PGF_{2\alpha}$

The two antisera were used in final dilutions of 1 20 000 1 50 000 these solutions binding about 50% of radioactive tracer (specific activity 60-160 Ci/mmol) Free and bound antigen were separated by precipitating the bound antigen with poly ethylene glycol according to van Orden and Farley (1973) The supernatant containing unbound radioactivity was dissolved in a scintillator based on touluene ethanol and Triton X 100^R and usually counted to 10 000 disintegrations Standard curves and cross reactions with various prostaglandins are summarized in Fig 2 for the $PGF_{2\alpha}$- and $PGF_{2\beta}$-antisera The dissociation constants and the concentration of binding sites in the two anti sera were estimated by Scatchard plots as illustrated in Fig 3 (cf Berson and Yalow 1959)

Comments on precision variability and specificity The radio immunoassays were found to have inter assay and intra assay va



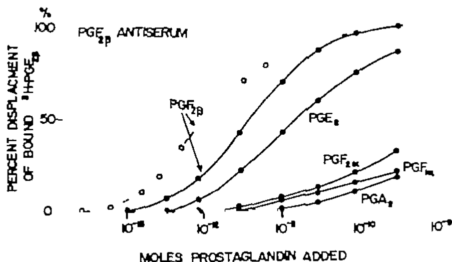


Fig 2 Standa d urves and ross-reactions of th $PGF_{2\alpha}$ antis rum (top) nd the $PGF_{2\beta}$-antis rum (bottom) with some p o t glandins Th two standard curves for $PGF_{2\beta}$ illustrate the use of ^{3}H-$PGF_{2\beta}$ with high nd low spe ific activity (about 150 and 60 Ci/mmol)

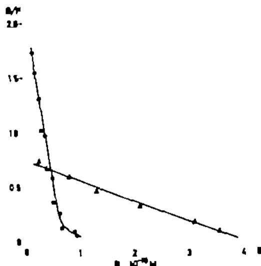


Fig 3 A plot of B/F v rsus B for the $PGF_{2\alpha}$-antiserum (1:50 000 ●—●) nd the $PGF_{2\beta}$-antiserum (1:21 000 Δ—Δ) where F den tes f e and B bound antigen Int - pretation according to $B/F = K(Ab_o - B)$ yield for the $PGF_{2\alpha}$-anti rum di sociation constant (K) equal t 3×10^{10} 1/mol and 7×10^{-11} M as the con ntration of binding ites (Ab_o) Corresponding figures f r the $PGF_{2\beta}$ anti- serum wer 2×10^{9} 1/mol and 4×10^{-10} M r spectively

riation coefficients in the range of 13 18% (I IV) To reduce the error due to inter-assay variation all urine samples from each ex periment were analyzed at one time and the results were in most cases expressed as per cent of control levels (cf Kindahl 1978)

There were several reasons for the intra assay variation The radio immunoassays were performed in five to seven sampling steps followed by centrifugation and decantation to separate bound and unbound ligand The standard curve were constructed by plotting ^{e}log (added PGF_2) on the abscissa versus fraction of displaced radio activity on the ordinate (cf Fig 2) If complete saturation of the antibodies is assumed (Cekan 1976 Shaw <u>et al</u> 1977) *the relative error of a determination on the ordinate corresponds* to a relative error on the abscissa which is at least four times larger (see Appendix) All unknown samples were analyzed in du plicate and the standard curve was based on triplicate analysis

as an approach to reduce this inherent dispersion

The problem of specificity was emphasized in the separate publications by referring to radioimmunoassay data as immunoreactive $PGF_{2\alpha}$ ($iPGF_{2\alpha}$) and $iPGE_2$ respectively For convenience this notation is not used here Attempts to increase the specificity of the radioimmunoassay by purification of the samples using organic solvents have led to problems with blank. (cf Kindahl 1978) PGE_2 and $PGF_{2\alpha}$ were therefore measured in unextracted rabbit urine In spite of the specificity of the antisera (cf Fig 2) known or unknown compounds with structural resemblance to $PGF_{2\alpha}$ and $PGF_{2\beta}$ could occur in concentrations sufficient to disturb the analyses of prostaglandins in rabbit urine It was therefore necessary to validate the radioimmunoassays

$PGF_{2\alpha}$ was measured by mass fragmentography and by radioimmuno assay in urine from six rabbits To reduce the interassay varia tion the radioimmunoassay was performed four times The mass fragmentography was run in duplicate as follows (Sjöquist et al unpublished) A 5 ml urine sample was extracted twice with chloroform at pH 3 and the dried chloroform extracts were evapo rated methylated and purified by preparative TLC (system ethyl acetate methanol water 80 13 50) The zone corresponding to the $PGF_{2\alpha}$ methyl ester (Rf 0 67) was eluted and derivatized as described above The mean deviation between the two methods was 13% demonstrating a reasonably good agreement (IV table I)

PGE_2 was measured by radioimmunoassay after reduction to $PGF_{2\alpha}$ and $PGF_{2\beta}$ This reduced the specificity of the method as dis cussed above for the mass fragmentographic analysis of PGE_2 in renal medulla However $PGF_{2\beta}$ is not naturally occurring in the rabbit The amount of PGE_2 in rabbit urine could be calculated as the sum of $PGF_{2\beta}$ and the increase in $PGF_{2\alpha}$ after reduction

This procedure was unsuitable since rabbit urine often contained more $PGF_{2\alpha}$ than PGE_2 The increase in $PGF_{2\alpha}$ was small compared with the level before reduction and could usually not be measu red with a high precision Instead the amount of $PGF_{2\beta}$ in the samples was used as a crude index of PGE_2

The radioimmunoassay for PGE_2 could not be validated as the $PGF_{2\alpha}$ radioimmunoassay because mass fragmentographic methods for analysis of PGE_2 in rabbit urine were not available Neverthe-less the urinary excretion of PGE_2 in rabbits was also measured by bioassay on rat fundus according to Weeks et al (1968) The values obtained by the two methods were of the same order of magnitude (IV)

Experimental

Male albino New Zealand rabbits were used for acute experiments The chronic studies were performed on female albino rabbits of the same strain

Choice of anesthetics The rabbits were anesthetized with chlora lose-urethane (I III VI) This method of anesthesia was chosen because Warren and Ledingham (1976) in a systematic study on the effects of different anesthetics on renal function demon strated that chloralose urethane had little influence on renal blood flow and sodium excretion in the rabbit

Analysis of plasma renin activity (PRA) PRA was measured by antibodies for angiotensin I essentially as described by Haber et al (1969) using a commercial kit obtained from SchwarzMann/ /BD Immunodiagnostics To reduce the inter assay variability data were often calculated in per cent of control in each experi ment

Comments on urine collection In acute experiments urine was collected from ureter cannulas The ureter of the rabbit is quite vulnerable and the cannulation sometimes resulted in a slight hematuria To reduce the contribution of prostaglandins from the cells in urine or from formed elements of blood indo methacin was added to the collecting vials which were also chilled In chronic experiments (IV V) 24 h urine specimens from rabbits in metabolic cages were collected in devices with dry ice to secure rapid cooling of the urine

Miscellaneous Arterial blood pressure and ureteral pressure were recorded with a Statham pressure transducer (P23Dc) on a Grass polygraph model 7B PAH inulin and creatinine were ana lyzed by spectrophotometry according to Smith et al (1945) Hilger et al (1958) and Slot (1965) respectively and clear ance was calculated by the standard formula

In vitro studies on 15 hydroxyprostaglandin dehydrogenases

The enzyme 15 hydroxyprostaglandin dehydrogenase (EC 1 1 1 141) was purified from swine kidneys by centrifugation (100 000xg) ammonium sulfate precipitation and Sephadex G 100 chromatography as described by Änggård and Samuelsson (1970) The purified en zyme preparation contained both type I (NAD^+ linked) and type II ($NADP^+$ linked) 15 hydroxyprostaglandin dehydrogenase activity The enzymes could be separated by affinity chromatography on $PGF_{2\alpha}$ agarose (Fig 4) The $PGF_{2\alpha}$ agarose was prepared by coupling $PGF_{2\alpha}$ to $H_2N\ (CH_2)_6$ agarose (AH Sepharose 4B Pharmacia Uppsala) using water soluble carbodiimide essentially as described for preparation of conjugates for immunization (I) The type II en zyme was not retained by the gel The type I e yme could be eluted by a shift in pH (Nagasawa et al 1

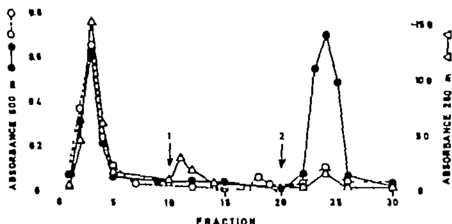


Fig 4 Separation of type I (NAD^+-linked) and typ II ($NADP^+$-linked) 15-hydroxyprostaglandin dehydrogenases by affinity hromatography on $PGF_{2\alpha}$-agarose The gel contained 2-4 µmol $PGF_{2\alpha}$/ml Th enzymes wer pplied in 3 ml followed by 30 ml 0 1 M potassium pho phate buffe pH 7 4 After wa hing with 0 5 M KCl in th same buffer (fi st arrow) lution f the typ I nzyme wa chieved by 0 1 M sodium acetate buffer pH 4 0 (s cond arrow) Absorb nce t 280 nm indicate p otein con cent tion and absorbance at 500 nm NAD^+ - and $NADP^+$-dependant enzyme activity

The effects of diuretics on the purified enzyme of type I were studied by incubating the enzyme PGE_2 (3x10 ^{4}M) NAD (2 5x10 ^{3}M) and the drug or solvent being tested in duplicate for 30 min at 37°C The amount of 15 keto PGE_2 formed was measured by spectrophotometry at 500 nm after alkali treatment as previously described (Änggård and Samuelsson 1970) The type II enzyme activity was measured in the same way but with NADP (2 5x10 ^{3}M) instead of NAD

Statistics

In papers I III and VI non parametric tests were employed These in cluded the sign test and Wilcoxon s rank sum test (Dixon and Massey 1957) In papers IV and V Student s t test was used In all cases a P value below 0 05 was considered significant

RESULTS AND DISCUSSION

Effects of nonsteroidal anti-inflammatory drugs on renal prostaglandin synthesis (I)

Nonsteroidal anti inflammatory drugs have been widely used to study the *in vivo* effects of a reduced renal prostaglandin biosynthesis (see Dunn and Hood 1977 for review) While these drugs effectively inhibit rabbit renal prostaglandin synthesis *in vitro* (Blackwell et al 1975 Pong and Levine 1976) their use as inhibitors *in vivo* has not been thoroughly investigated Differences in distribution and in the rate and route of elimination of the drugs might thus invali date *in vitro* observations (Brune et al 1976) It was therefore necessary to characterize dose and time-response relationships for inhibition of renal prostaglandin synthesis For this purpose four drugs were chosen aspirin diclofenac sodium indomethacin and na proxen all of which have previously been found to reduce prosta glandin synthesis in *in vitro* systems (Tomlinson et al 1972 Ku et al 1975 Blackwell et al 1975 Pong and Levine 1976)

The *in vivo* effects of the four drugs were studied on the concen tration of PGE_2 and $PGF_{2\alpha}$ in the rabbit renal medulla The prosta glandins were measured by mass fragmentography At five minutes *post mortem* the accumulated levels of PGE_2 and $PGF_{2\alpha}$ in the medulla untreated rabbits were $9\ 2 \pm 2\ 2$ (S D) and $1\ 5 \pm 0\ 6$ µg/g wet weight respectively These amounts are greater than the *in vivo* levels since prostaglandins accumulate rapidly in tissues after death (van Dorp 1971 Änggård et al 1972) These high levels of prostaglan dins were considered suitable as a starting point for studying the inhibitory effects of the four drugs on prostaglandin synthesis

The four drugs reduced PGE_2 and $PGF_{2\alpha}$ synthesis in a dose-dependent manner and their levels in the renal medulla decreased in parallel Thirty min after i v drug administration the ED_{95} for inhibition

of renal prostaglandin synthesis was 15 mg/kg of aspirin 1 5 mg/kg of diclofenac sodium 1 5 mg/kg of indomethacin and 5 mg/kg of naproxen

The duration of inhibition of prostaglandin synthesis after a dose twice the ED_{95} was estimated by following the urinary excretion of PGE_2 and $PGF_{2\alpha}$ by radioimmunoassay The depression of urinary prosta glandin excretion lasted for up to three hours The urinary excretion of PGE_2 and $PGF_{2\alpha}$ was however not completely reduced in spite of this high dose The inhibition seldom exceeded 95% indicating that higher doses may be needed to completely decrease prostaglandin ex cretion *in vivo*

In subsequent *in vivo* experiments aspirin diclofenac sodium and indomethacin were used in doses twice ED_{95} in order to study the renal effects of prostaglandin synthesis inhibition (II V VI) It was no ticed in these experiments that the inhibition of urinary prostaglan din excretion was not complete and usually ranged between 70 and 95%

There are few comparative studies on inhibition of renal prostaglandin synthesis after *in vivo* administration of nonsteroidal anti inflam matory drugs Gafni et al (1978) compared the potency and duration of aspirin indomethacin and meclofenamic acid on the prostaglandin synthesis in rabbit renal medulla *in vivo* Their results for indo methacin and aspirin are similar to the results above They found in domethacin (1 mg/kg) and aspirin (10 mg/kg) to be the lowest dose producting a maximal degree (92 98%) of prostaglandin synthesis in hibition The maximal inhibition lasted 2 h for indomethacin and 6 h for aspirin

The present results and the study by Gafni et al (1978) indicate the doses of aspirin like drugs that are likely to reveal the possible physiological effects of a reduced renal prostaglandin synthesis These drugs can reduce prostaglandin synthesis at doses which are lower than those needed to elicit many other pharmacological effects (Vane

1978) The use on nonsteroidal anti inflammatory drugs at a dose twice ED_{95} might therefore be justified However results obtained with aspirin like drugs should be interpreted with caution when used to in fer the functional importance of prostaglandins (Flower 1974 Dunn and Hood 1977) Such conclusions are likely to be more justified if stu dies with structurally different inhibitors of prostaglandin synthe sis give similar results

6 keto prostaglandin $F_{1\alpha}$ in rabbit kidney (V)

6 keto $PGF_{1\alpha}$ was demonstrated in rabbit renal medulla and cortex by mass spectrometry (V) The *post mortem* accumulation of 6 keto $PGF_{1\alpha}$ was measured by mass fragmentography in renal cortex medulla and pa pilla The cortex contained 1 4 $^+$ 0 3 (S E n 9) the medulla 2 1 $^+$ 0 3 (n=8) and the papilla 3 7 $^+$ 0 7 (n=4) µg/g wet weight 6 keto $PGF_{1\alpha}$ is formed from PGI_2 by hydrolysis (Cho and Allen 1978) The demonstration of large amounts of 6 keto $PGF_{1\alpha}$ in the kidney in dicates a considerable synthesis of PGI_2

The level of 6 keto $PGF_{1\alpha}$ increased from the renal cortex to the papilla Accumulation of other prostaglandins has also been found to be most abundant in medulla and papilla (cf Änggård et al 1972 Larsson and Änggård 1976) The difference in prostaglandin synthesis between cortex and medulla could be due to the availability of en zymes substrate cofactors and catabolism or release of prostaglan dins to blood and urine

The levels of 6 keto $PGF_{1\alpha}$ could be compared with the accumulated le vels of PGE_2 and $PGF_{2\alpha}$ in the rabbit kidney Under similar experimen tal conditions the renal medulla contained 9 2 $^+$ 2 2 (S D) µg/g of PGE_2 and 1 5 0 6 µg/g of $PGF_{2\alpha}$ (I) PGE_2 is thus quantitatively more important than $PGF_{2\alpha}$ and 6 keto $PGF_{1\alpha}$ in the medulla

In the cortex the accumulation of 6 keto $PGF_{1\alpha}$ is quite large in com parison with the reported levels of PGE_2 and $PGF_{2\alpha}$ Larsson and Äng

gård (1976) found that the cortex of the rabbit kidney contained about 0 2 µg/g of PGE_2 and $PGF_{2\alpha}$ These results indicate that relative to PGE_2 and $PGF_{2\alpha}$ the formation of PGI_2 and 6 keto $PGF_{1\alpha}$ might be more important in the cortex This is supported by a recent study of Whorton et al (1978) demonstrating that rabbit cortical microsomes produce significant amounts of 6 keto $PGF_{1\alpha}$ from radioactive arachidonic acid or PGG_2 while medullary microsomes produced almost exclusively PGE_2

The renal cells which contain prostacyclin synthase are not known It might be expected that the enzyme is located in close connection to prostaglandin endoperoxide synthase The latter enzyme has been found particularly in the collecting ducts of the renal medulla (Janszen and Nugteren 1971 Smith and Wilkin 1977) However prostacyclin synthase was originally discovered in vascular walls (Moncada et al 1976) It seems possible that 6 keto $PGF_{1\alpha}$ in the renal cortex originates from PGI_2 formed by the vascular endothelium Many effects of nonsteroidal anti inflammatory drugs and arachidonic acid were previously attributed to effects on prostaglandin synthesis in particular on formation of PGE_2 in the kidney (McGiff et al 1974 Larsson and Änggård 1974) The relatively large cortical synthesis of 6 keto $PGF_{1\alpha}$ indicates that inhibition of PGI_2 synthesis also might account for some of the effects of nonsteroidal anti inflammatory drugs on renal blood flow and renin release (Whorton et al 1977 Gerber et al 1978a)

Renal prostaglandin synthesis and renal function

Earlier studies indicated that prostaglandins could affect renal handling of sodium although their exact role was unsettled Acute and chronic changes in sodium balance are compensated by partly different homeostatic mechanisms (eg Carey et al 1976) It was therefore of interest to study renal prostaglandins during acute changes in extracellular fluid volume and during chronic changes in sodium intake

Relation to acute changes in extracellular fluid volume (II III)

Volume expansion (II) An acute extracellular fluid volume expansion with saline increases urinary sodium and water excretion which is often referred to as saline diuresis The present study was performed to investigate how saline diuresis would affect urinary prostaglandin excretion and how inhibitors of prostaglandin synthesis would affect the saline diuresis

An acute expansion of the extracellular fluid volume with Ringer solu tion (50 ml/kg/h for 1 h) in anesthetized rabbits enhanced sodium and water excretion it depressed plasma renin activity and reduced the urinary excretion of $PGF_{2\alpha}$ by over 50% (II) The changes in PGE_2 ex cretion were inconsistent Pretreatment with indomethacin and diclo fenac sodium reduced urinary excretion of PGE_2 and $PGF_{2\alpha}$ by 75 95% but enhanced the saline diuresis in comparison with the control group These results suggested that during saline diuresis following volume expansion the renal prostaglandin synthesis might be reduced and that further reduction by indomethacin and diclofenac sodium augmented the urinary salt and water excretion

These effects of indomethacin and diclofenac sodium on urine flow and sodium excretion during saline diuresis were unexpected These drugs usually reduce sodium excretion in anesthetized animals (Zins 1975) This effect was also noted before the volume expansion (II cf VI) The variable effects might be explained by differences in fluid and electrolyte balance

Indomethacin and diclofenac sodium reduced renal blood flow but this effect was abolished during volume expansion (II) One explanation for the increased sodium excretion during extracellular fluid volume expansion might therefore be that the effects of the drugs on renal blood flow were counteracted pointing to a natriuretic effect of prostaglandin synthesis inhibition as proposed by Kirschenbaum and Stein (1976 1977)

Kirschenbaum and Stein (1976) found that meclofenamate a prostaglan din synthesis inhibitor increased sodium excretion in awake dogs un dergoing water diuresis During extracellular fluid volume expansion these workers demonstrated that the renal effects of indomethacin and meclofenamic acid could be explained by a decreased tubular reabsorp tion of sodium (Kirschenbaum and Sten 1977) These and other observa tions indicate an antinatriuretic role of prostaglandins in the kidney.

It is possible that effects of anesthetics might explain the contra dictory results which have emerged In anesthetized rats and dogs so dium excretion and saline diuresis were not augmented by inhibitors of prostaglandin synthesis (Leysack et al 1975 Susic and Spark 1975 Bohan and Wesson 1976 Feigen et al 1976 cf Kirschenbaum and Stein 1977) The effects of nonsteroidal anti inflammatory drugs on renal blood flow are increased in anesthetized animals (Zins 1976) It is therefore likely that the effects of these drugs on renal function de pend on the experimental conditions such as type of anesthesia as well as fluid and electrolyte balance

Volume contraction caused by furosemide (III) This study was perfor med to investigate the effects of an acute extracellular volume con traction on the urinary prostaglandin excretion This was achieved with furosemide a potent loop diuretic

A constant infusion of furosemide (0 13 mg/min for 1 h)to anesthetized rabbits increased sodium excretion tenfold potassium and PGE_2 excre tion threefold and resulted in a substantial loss of sodium and water (III) The increase in PGE_2 excretion occurred during the furosemide diuresis and could thus be related to the pharmacological effects of furosemide e g on sodium excretion renin release or on renal blood flow

The furosemide diuresis was followed by a large increase in urinary $PGF_{2\alpha}$ excretion The increase occurred when the diuretic effect of furosemide had terminated indicating that it was not related to

the furosemide induced losses of salt and water

*Furosemide has previously been found to increase renal venous secre*tion of PGE_2 in dogs (Williamson et al 1975) Furosemide also in crease urinary excretion of PGE_2 and $PGF_{2\alpha}$ in humans (Abe et al 1977 Weber et al 1977a Scherer et al 1978) although the temporal dif ference between the excretion of PGE_2 and $PGF_{2\alpha}$ noticed above have not been previously reported

The mechanism of the increase in renal excretion and secretion of prostaglandins following furosemide is unknown Weber et al (1977a) proposed that furosemide increased the urinary excretion of $PGF_{2\alpha}$ by raising the plasma concentration of arachidonic acid and thus stimu lating the renal prostaglandin synthesis This proposition could not account for the temporal difference between the effects of furosemide and the increase in $PGF_{2\alpha}$ excretion Abe et al (1977) proposed that furosemide increased urinary prostaglandin excretion by inhibiting *the enzyme 15 hydroxyprostaglandin dehydrogenase type I in the kidney* Diuretic drugs have previously been found to inhibit this enzyme in placenta *in vitro* (Paulsrud and Miller 1974) but not *in vivo*

An *in vitro* study on the effect of furosemide and related drugs on the enzyme activity of 15 hydroxyprostaglandin dehydrogenase type I from swine kidney was performed This study was referred to as un published observations in III The results are summarized below

Diuretics	Per cent inhibition		I_{50}
	0 1 mM	0 1 mM	(mM)
Bumetanid	0 - 4%	0 - 4%	-
Ethacrynic acid	22 ± 5%	47 ± 6%	1 5
Furosemide	7 2%	41 ± 5%	1 9
Polyth azide	6 ± 5%	29 ± 5%	4 0
Hydrochlorothiazid	0 - 4%	0 - 4%	-
Theophylline	0 - 4%	0 - 4%	

Although many diuretics are excreted and concentrated in urine the relevance of inhibitory concentrations in the millimolar range is not clear and needs to be confirmed *in vivo*

The effect of furosemide was also tested on the type II 15 hydroxy prostaglandin dehydrogenase *in vitro* Furosemide (1 mM) did not in hibit the en yme The possibility that furosemide increases urinary prostaglandin excretion by inhibiting 15 hydroxyprostaglandin dehydro genase enzymes must therefore be considered unlikely (cf Gerber et al 1978c)

In summary two inhibitors of prostaglandin synthesis increased the diuretic effects of an acute extracellular volume expansion with Ringer solution The expansion was also followed by a decreased urina ry $PGF_{2\alpha}$ excretion An acute extracellular volume contraction caused by furosemide was followed by an increase in $PGF_{2\alpha}$ excretion The furo semide diuresis was also accompanied by a relatively small increase in PGE_2 excretion indicating that renal prostaglandins might also be directly influenced by furosemide

Relation to chronic changes in sodium intake (IV V)

Acute and chronic changes in sodium balance are compensated for by partly different mechanisms (Carey et al 1976) The results above indicated that prostaglandins could be involved in the renal homeo stasis of acute changes of extracellular fluid volume Renal prosta glandin synthesis is also affected by anesthetics (Zins 1976) The effects of chronic changes in intake of sodium chloride on urinary prostaglandin excretion were therefore studied in *unanesthetized rab bits*

Female rabbits in metabolic cages were found to excrete significantly more PGE_2 (IV) and PGF_{α} (IV V) when they were fed a low salt diet (0 05-0 4% NaCl) than when they were given a corresponding diet with more salt (2 NaCl or more) The changes in PGE_2 and $PGF_{2\alpha}$ excretion

did not seem to depend on changes in water consumption or on any other dietary differences (V)

The inverse relationship between the urinary excretion of sodium and prostaglandins indicated that the renal synthesis of prostaglandins is influenced by sodium intake Similar observations have been made earlier Leary et al (1974) found an impaired relea e of prostaglan dins from kidneys of saltloaded rats Tobian and O Donnell (1976) found the level of PGE_2 in the medulla of the rat kidney to be in versely related to sodium intake The urinary excretion of PGE_2 has also been found to be inversely related to sodium intake (Weber et al 1977b Scherer et al 1978) but lack of such a relationship has also been reported (Lifschitz et al 1978)

The enhanced urinary excretion of prostaglandins in rabbits on a so dium restricted diet suggests that prostaglandins could be related to sodium conserving mechanisms An antinatriuretic action of prosta glandins in the kidney was proposed by Tobian and O Donnell (1976) and by Kirschenbaum and Stein (1976 1977) This hypothesis is in accor dance with the effects of prostaglandin synthesis inhibitors on sodium excretion following an acute expansion with Ringer solution (II) Inhibition of prostaglandin synthesis could also be expected to in crease sodium excretion in chronically sodium deprived rabbits It was found that two days treatment with aspirin (30 mg/kg x 2) did not in crease urinary sodium excretion significantly while the excretion of $PGF_{2\alpha}$ was reduced by 85% (V) This unexpected finding is in agree ment with a recent study by Lifschitz et al (1978) and does not sup port a major role of prostaglandins in the chronic regulation of so dium balance in the kidney However the renal prostaglandins might be related to other mechanisms which are influenced by acute or chronic changes in fluid and electrolyte balance such as renin release (cf Weber et al 1977b)

The urinary excretion of 6 keto $PGF_{1\alpha}$ was not significantly changed by a 300 fold increase in urinary sodium excretion from

0 09 mmol Na^+/day to 27 5 mmol Na^+/day after addition of salt (V) This finding might indicate that renal synthesis of PGI_2 is not in fluenced by chronic variations in sodium intake Another possibility is that the 6 keto-$PGF_{1\alpha}$ detected in urine to a large extent origina tes from PGI_2 produced elsewhere in the body PGI_2 might be a circulating hormone (Moncada et al 1978 Waldman et al 1978) which is 40% extracted during circulation through the kidneys (Gerkens et al 1978)

In summary urinary excretion of PGE_2 and $PGF_{2\alpha}$ was influenced by chronic changes in sodium intake in a way which suggests a connection with sodium conserving mechanisms such as the renin angiotensin system

Prostaglandins and the renin angiotensin system (II IV,V VI)

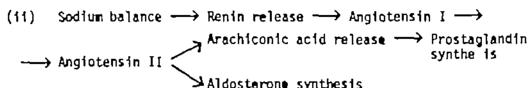
Two alternative mechanisms

Renin release is increased by sodium deficiency and depressed by so dium loading (Davis and Freeman 1976) The urinary excretion of PGE_2 and $PGF_{2\alpha}$ during variations in sodium intake indicate that renal prostaglandin synthesis and renin release change in parallel

Many observations indicate that prostaglandins could be involved in renin release mechanisms Arachidonic acid the prostaglandin pre cursor acid has been found to increase renin release both *in vivo* (Larsson et al 1974 Bolger et al 1976) and *in vitro* (Weber et al 1976) Prostaglandin endoperoxides and PGI_2 also increase renin re lease *in vitro* (Weber et al 1976 Whorton et al 1977) Conversely inhibitors of prostaglandin synthesis has been found to reduce renin release both *in vitro* and in many *in vivo* situations (Larsson et al 1974 Weber et al 1976 Romero et al 1976 see Dunn and Hood 1977 for review) These studies support an effect of renal prostaglandin synthesis on renin release possibly according to the following scheme

(i) Arachidonic acid release ⟶ Prostaglandin synthesis ⟶ Renin release ⟶ Angiotensin I ⟶ Angiotensin II ⟶ ⟶ Aldosterone synthesis

There might however be an alternative explanation to the parallel changes in urinary prostaglandin excretion and the activity of the renin angiotensin system Renal prostaglandin synthesis could be in fluenced by angiotensin II according to the following scheme



Angiotensin II has thus been found to increase renal prostaglandin synthesis *in vitro* (Danon et al 1975) and to increase urinary prostaglandin excretion (Frölich et al 1975 Dunn et al 1978) An increased renal synthesi of vasodilating prostaglandins following angiotensin II has also been proposed to defend the renal circulation against its vasoconstrictor effect (Aiken and Vane 1973 McGiff et al 1974) The variations in urinary prostaglandin excretion could be explained by either of the above two mechanisms although it is obvious that the two mechanisms cannot operate at the same time Prostaglandin synthesis and renin release could in this case be expected to mu tually reinforce each other and increase beyond all limits

Prostaglandin and renin-angiotensin interactions in an acute ureteral occlusion (VI)

The proposed mechanisms (i) and (ii) discussed above were experi mentally tested using an acute unilateral ureteral occlusion as a model for prostaglandin and renin angiotensin interactions An acute ureteral occlusion releases renin which in turn leads to formation of endogenous angiotensin II (Eide et al 1977)

According to proposition (i) prostaglandins could be important for the release of renin This was studied during an acute ureteral occlu sion using diclofenac sodium to inhibit prostaglandin synthesis

According to proposition (ii) endogenous angiotensin II could in crease the pro taglandin synthesis of the contralateral kidney This

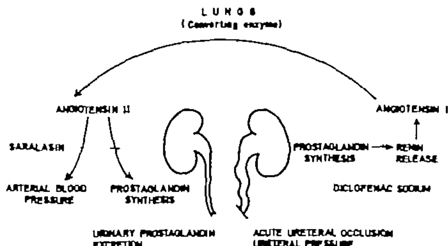


Fig 5 An acut unilat al ur t al cclusion as a model for pr staglandin and renin angiotensin int actions(se t xt fo xplanati ns)

was studied by following the urinary excretion of prostaglandins from this kidney and by using saralasin to block the effects of endogenous angiotensin II The experimental model is illustrated in Fig 5

In the control group the unilateral ureteral occlusion increased the arterial blood pressure (11 $^{+}$ 5 mm Hg Fig 6) the plasma renin acti vity (3 5 times) and the urinary excretion of prostaglandins (4 5 times) in the contralateral kidney The elevation of plasma renin ac tivity indicated that angiotensin II could be responsible for the in crease in arterial blood pressure and for the increase in urinary excretion of prostaglandins This hypothesis was tested by infusion of saralasin at a rate which blocked the pressor effect of exogenous an giotensin II

During infusion of saralasin the increase in arterial blood pressure following the ureteral occlusion was blocked although the increase in urinary prostaglandin excretion was unaffected These results indi cated that angiotensin II was of importance for the increase in arte rial blood pressure In this case the increase in urinary prostaglan

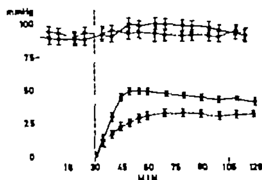


Fig 6 Mean arterial blood pressure and mean ureteral pressure foll wing an acute ur teral occlusion at the dotted lin (30 min) Contr ls (n=6 •——•) and diclofenac sodium pretreated group (3 mg/kg n=6 o——o) The ur teral pressure of the latt r group was significantly reduc d A similar ffect of indomethacin on the ureteral pressure in d gs was recently reported by Allen et al (1978)

din excretion could be due to activation of other factors besides angiotensin II

Pretreatment with diclofenac sodium abolished the increase in plasma renin activity after ureteral occlusion and blocked the increase in arterial blood pressure (cf Fig 6) The results support the con cept that prostaglandins might be important for renin release and that renin angiotensin in turn mediates the increase in arterial blood pressure These findings are in agreement with proposition (i)

Diclofenac sodium reduced the pressure of the occluded ureter in comparison with the control group (Fig 6) indicating that prostaglandin ynthesis inhibition enhanced the resistence of the afferent arterioles The afferent arterioles play an important role in the barorecep tor control of renin release (Davis and Freeman 1976) The effect of diclofenac sodium on renin release and on renal blood flow may there fore be closely interrelated

Renin release is regulated by at least two major intra renal receptor mechanisms (Davis and Freeman 1976)

(1) The renal baroreceptor of the afferent arterioles

(2) The sodium chloride sensitive *macula densa* receptor of the di tal tubules Both receptors could be activated by an acute ureteral occlusion

Ad (1) After an acute ureteral occlusion there is a paradoxical vaso dilatation in the kidney (Murphy and Scott 1966 Eide et al 1977) which might increase renin release by the baroreceptor mechanism The vasodilatation can be blocked by inhibitors of prostaglandin synthesis (Olsen et al 1976 Allen et al 1978) The reduced renin secretion following diclofenac sodium indicated that prostaglandins might be involved in the baroreceptor control of renin release (cf Data et al 1978)

Ad (2) An acute ureteral occlusion reduces the amount of sodium reaching the *macula densa* This might stimulate renin release by the *macula densa* receptor mechanism (Eide et al 1977) It is however less likely that diclofenac sodium reduced renin release by this mechanism The drug reduced the ureteral pressure and thus less sodium was filtrated in the glomeruli Diclofenac sodium would there fore be expected to increase rather than decrease renin release by this mechanism The renal vasodilatation and the increase in renin release could be explained by an increased prostaglandin synthesis following an acute ureteral occlusion Experimental evidence indicates that this might be the case Olsen et al (1976) and Olsen 1978 found that a rise in intrarenal pressure augmented the urinary excretion of prostaglandins from the affected kidney The effects of diclofenac sodium on renin release on ureteral pressure and on renal blood flow could therefore be attributed to inhibition of prostaglandin synthesis in the renal cortex

The renal cortex was found to form relatively large amounts of 6 keto $PGF_{1\alpha}$ (V) indicative of an important synthesis of PGI_2 in this re gion (Whorton et al 1978) PGI_2 is a potent renal vasodilator (Friesinger et al 1978) It increases renin release both *in vivo* and *in vitro* (Whorton et al 1977 Gerber et al 1978a) The pre sent study indicates a possible role of cortical prostaglandins in regulation of renin release and renal blood flow Prostaglandins might increase renin release either by a direct action on the renin-contain-

ing cells or indirectly by renal vasodilation and the baroreceptor mechanism These effects of cortical prostaglandins on renal blood flow and renin release are likely to be closely linked

Prostaglandins in the kidney might influence the renal handling of sodium and water via effects on renal blood flow on the renin angio tensin aldosterone system or directly on the renal tubular cells The renal circulatory effects of prostaglandins could promote sodium and water excretion eg by opposing renal vasoconstriction It might also be argued that prostaglandins could cause sodium retention by stimula ting the renin angiotensin aldosterone axis Finally prostaglandins might have tubular effects as proposed by many other investigators It seems that the possible tubular effects of prostaglandins are diffi cult to experimentally separate from their other renal actions The present series of experiments indicate that under certain experimen tal conditions renal prostaglandins might be involved in sodium and water retaining mechanisms indirectly via stimulation of the renin angiotensin aldosterone system and directly by the possible tubular actions of prostaglandins

CONCLUSIONS

The prostaglandin system of the rabbit kidney was studied with emphasis on the regulation of acute and chronic changes in sodium balance the renin angiotensin system and the effects of diuretics The results were achieved by a combination of pharmacological and biochemical methods

1 PGE_2 was quantitatively the most important prostaglandin in the renal medulla while 6 keto $PGF_{1\alpha}$ the stable product of PGI_2 was quantitatively the most important prostaglandin in the cortex
2 The dose- and time response relationships for inhibition of renal prostaglandin synthesis *in vivo* were determined for aspirin diclofenac sodium indomethacin and naproxen The results were used in subsequent experiments
3 Diclofenac sodium and indomethacin enhanced the saluretic and diuretic effects of an acute extracellular volume expansion with Ringer solution in anesthetized rabbits
4 Urinary $PGF_{2\alpha}$ excretion was decreased by an acute volume expan sion caused by Ringer solution and it was increased by an acute volume contraction caused by furosemide Furosemide also in creased urinary PGE_2 excretion which might be related to the renal vasodilating action of this drug
5 Chronic sodium loading in conscious rabbits decreased the urinary PGE_2 and $PGF_{2\alpha}$ excretion while chronic sodium deficiency enhan ced the urinary PGE_2 and $PGF_{2\alpha}$ excretion These results suggested a connection between renal prostaglandins and renal control of sodium balance and in particular with the renin angiotensin system
6 The relations between the renal prostaglandins renin release and the action of angiotensin II were studied during an acute unilate ral occlusion The occlusion increased plasma renin activity (PRA), the arterial blood pressure and the urinary prostaglandin excre tion from the contralateral kidney Infusion with saralasin an angiotensin II antagonist blocked the increase in arterial blo-

pressure but not urinary prostaglandin excretion suggesting that other mechanisms besides angiotensin II may be involved in regulation of renal prostaglandin synthesis Pretreatment with diclofenac sodium blocked the increase in PRA and arterial blood pressure and reduced the pressure in the occluded ureter The results indicate a role of prostaglandins in regulation of renal blood flow and renin release Renal prostaglandins might increase renin release either by a direct action on the renin containing cells or indirectly by renal vasodilatation and the baroreceptor mechanism

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A P P E N D I X

> I could have done it in a much more complicat d way said the red Queen immensely proud
>
> Lewis Carroll

Comments on the reliability of radioimmunoassays

The radioimmunoassays for prostaglandins were found to have a rather large dispersion although in the same range as reported for other assays (eg Cekan 1976 Kindahl 1978) Since this problem might be generally encountered it seemed justified to briefly di cuss the intra assay variability In the following it is assumed that the anti sera are saturated with tracer and ligand the so called model of satu *ration analysis (Cekan 1976 Shaw et al 1977) and that tracer and* ligand have the same affinity for the antibodies

Abbreviations

B(x) denotes bound tracer (radioactivity or absolute amount) when x non labelled ligand is added F(x) denotes free tracer The total amount of tracer is $T = B(x) + F(x)$ Logit x $^e\log\{x (1 x)\}$ tanh x (exp 2x 1) (exp 2x + 1) and Φ the normal distribution function

Sampling statistics

Let the number of samples be N and let each sample be assayed in n plicate Let $F^1(x)$ $F^2(x)$ $F^n(x)$ be the radioactivity of the un bound tracer in a sample with x ligand $F^i(x)$ is subject to errors of pipetting separation of free and bound tracer counting of radioacti vity etc If all $F^i(x)$ are counted to a fixed number of disintegra tions it may then be reasonable to assume that all $F^i(x)$ are normally distributed independent stochastic variables with the same mean dependent on x and the same variance independent of x (see below)

$F(x)$ is transformed to the fraction of displaced tracer by subtracting F(0) and dividing with B(0) i e by setting $Y^i(x)$ $\{F^i(x)$ $F(0)\}$ B(0) Let $\bar{Y}(x)$ be the arithmetic mean of $Y^i(x)$ i 1 2 n It follows that $\bar{Y}(x)$ is normally distributed with a mean $\mu(x)$ and a standard devia $\sigma n^{\frac{1}{2}}$ The standard curve is used to obtain x from $\bar{Y}(x)$ of an un known sample

Standard curve

Standard curves for radioimmunoassays may be constructed in many ways eg by plotting F(x) B(x) or B(x) F(x) versus x or log x (Thorell and Larson 1978) Rodbard et al (1968 1976) proposed the logit transfor mation of B(x) B(0) plotted versus log x In paper I standard curves were obtained by plotting the fraction of displaced tracer versus log x This method will be discussed here and its relation to the logit transformation will be derived

A complete saturation of the antiserum with tracer implies that the fraction d of bound tracer which is displaced by x ligand is equal

to (Shaw et al 1977)

(1) $$d(x) \quad \frac{x}{x+T}$$

Introducing t log x in (1) gives

(2) $$d(\,^{t})=u(t)=\frac{e^{t}}{e^{t}+T}$$

A plot of u(t) versus t would be expected to give the familiar sigmoidal log dose response curve which is shown by the $PGF_{2\alpha}$ and $PGF_{2\beta}$ standard curves in Fig 2 (page 18) The standard curves are symmetrical about the midpoint at which 50% of bound tracer is displaced and at which the maximum slope occurs The assays could therefore be expected to have the highest precision in this region

In paper I the middle part of the standard curve was approximated by a straight line obtained by the least square method This is justified by the rapid convergence of the series expansion of u(t) for t near the midpoint log T

(3) $$u(t)=\tfrac{1}{2}+\tfrac{1}{4}(t-\log T)-\tfrac{1}{48}(t-\log T)^{3}+\tfrac{1}{480}(t-\log T)^{5}+$$

It follows that the two first terms in (3) are a good approximation of u(t) about the midpoint The corresponding approximation of u(t) for t near t_0 $u(t_0)$ μ will be used later

(4) $$u(t)\approx\mu+u'(t_0)(t-t_0)=\mu+\mu(1-\mu)(t-\log T-\log\{\mu\ (1\ \mu)\})$$

To obtain a linear dose response relationship u(t) is rewritten by help of the hyperbolic function tanh

(5) $$u(t)=\tfrac{1}{2}\quad\tfrac{1}{2}\tanh\{\tfrac{1}{2}(t-l\ g\ T)\}$$

A plot of u(t) versus tanh $\{\frac{1}{2}(t \log T)\}$ would be expected to give a straight line if the saturation model is applicable to the antiserum in question Fig 1A illustrates such a plot of a $PGF_{2\alpha}$ standard curve indicating a linear relationship

The inverse of the standard curve is used to obtain x or log x from $\bar{Y}(x)$ of an unknown sample The inverses of (1) and (2) are respectively

(6) $$x=dT:(1-d)$$

(7) $$t\ \log x=\log\{Tu:(1-u)\}$$

The stochastic variables $\bar{X}$ and $\bar{T}$ log $\bar{X}$ are defined by substituting d and u with the stochastic variable $\bar{Y}$ It should be noted that (7) is equivalent to the logit transformation of Rodbard et al (1968 1976)

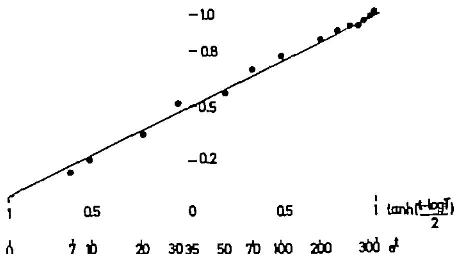
Reliability

The distribution of $\bar{T}$ is obtained from (7)

(8) $$P(T<\xi)=P(\bar{Y}<\ \,^{\xi}:(\,^{\xi}\ T))=\Phi\{(\tfrac{1}{2}+\tfrac{1}{2}\tanh\{\tfrac{1}{2}(\xi-\log T)\}-\mu)\ \sigma n^{-\frac{1}{2}}\}$$

where μ and $\sigma n^{-\frac{1}{2}}$ are the mean and the standard deviation of $\bar{Y}$ Conversely

(9) $$P\{\bar{Y}<\eta\}=P\{T<l\ g(T\eta:1-\eta)\}=P\{\bar{X}<T\eta:(1-\eta)\}$$



F g 1A. A standard curve of th $PGF_{2\alpha}$-radioimmunoassay The fraction of displaced radioactivity (on the rdinate) was plotted versus tanh {½(t-log T)} where t is the natural logarithm of added non-labelled $PGF_{2\alpha}$ and where T is the amount of ligand which displaced 50% of bound tracer T was estimat d from the mid-point of the standard curve The amount of added non-labell d ligand in pg is indicat d on the exp t scale below the abscissa Each sample w s counted to 10 000 disintegrations and the point represent mean of triplicate analyses According to the saturation model th points should b expect d to fall on the oblique line in the figure

Let s^2 be the pooled variance i e $s^2\ N^{-1}(n-1)^{-1}\Sigma\Sigma\ (Y^i(x_k)-\bar{Y}(x_k))^2$ Noting that $(\bar{Y}\ \mu)\ sn^{\frac{1}{2}}$ has a t distribution with N(n 1) degrees of freedom (Cramér 1946) confidence intervals for $\bar{T}$ are obtained from the distribution of $\bar{Y}$ by (9) From the radioimmunoassay data in Fig 1A s was found to be 0 0327 This figure was used in Table 1A to predict 95% confidence intervals for measurements at three points of the standard curve

Table 1A is based on the assumption of equal variance Rodbard et al (1976) have pointed out that the variance of the response variable is not constant at different dose levels for many radioimmunoassays This possibility was checked for the $PGF_{2\alpha}$ radioimmunoassay by 12 replicate analysis at three different dose levels at zero dose near the midpoint and close to the maximal dose All samples were counted to 10 000 disintegrations The observed variances were not significantly changed from the zero (s^2 1 5x10 ^{3}) to the maximal dose level (s^2 1 2x10 ^{3}) The variance was therefore assumed to be constant

Table 1A Predicted 95% confidence intervals for the determinations by the $PGF_{2\alpha}$-radioimmunoassay when each sample is assayed 2 3 4 or 9 times

Fraction displaced tracer (μ)	$\bar{X}$ $T\mu:(1-\mu)$ **	Predicted 95% confidence intervals for $\bar{X}$ * n 2	n=3	n=4	n=9
0 20	8 75 pg	(6 3 11 5)	(6 7 11)	(7 0 10 7)	(7 6 10)
0 50	35 pg	35 ± 7 3	35 ± 5 9	35 ± 5 0	35 ± 3 3
0 80	140 pg	(106 194)	(112 182)	(115 175)	(122 162)

* Data alculated from $\bar{Y} = \mu \pm sn^{-\frac{1}{2}}t_{.975}$ by help of (9) with -0 0327
** T=35 pg cf Fig 1A

Table 1A and the following approximation (eg Chang et al 1975) which follows from (4) or (7) if $\sigma n^{-\frac{1}{2}}$ is small in comparison with μ and $1-\mu$ illustrate that the variance of the final determinations is not constant

$$(10) \qquad \bar{T} = \log T + 1\ g(\bar{Y}\ (1-\bar{Y})) \approx \log T + \log(\mu:(1-\mu)) + \frac{\bar{Y}-\mu}{\mu(1-\mu)}$$

$\bar{T}$ is thus approximately normally distributed with mean $\log(T\mu\ (1\ \mu))$ and standard deviation $\sigma n^{-\frac{1}{2}}\mu^{-1}(1-\mu)^{-1}$ Many investigators restrict their measurements to the interval $0\ 2 < \mu < 0\ 8$ in which case the standard deviation of $\bar{T}$ varies approximately between 4 and 6 25 times $\sigma n^{-\frac{1}{2}}$

Summary

The above results demonstrate the intra assay variability of a radio immunoassay for $PGF_{2\alpha}$ and illustrate how the variability could be expected to decrease with the number of determinations of each sample in different regions of the standard curve In many biological appli cations of radioimmunoassays only relatively large changes in the con centration of measured hormones are of real biological significance It is therefore often sufficient with duplicate analysis of each sample (cf Chang et al 1975)

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 462

Physical performance, skeletal muscle enzyme activities, and fibre types in monozygous and dizygous twins of both sexes

Appendix by
PAAVO V KOMI and JAN KARLSSON

STOCKHOLM 1979

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 462

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Dedicated to the late professor Erkki Karvinen who inspired and encouraged us in the twin studies

CONTENTS

This study has partly been presented in the following papers:

1 KOMI P V J.H.T VIITASALO M. HAVU A. THORSTENSSON and J KARLSSON Physiological and structural performance capacity: effect of heredity In: Biomechanics V-A. (Ed. P V Komi) University Park Press Baltimore 1976 pp 118-123

2 KOMI P V J H T VIITASALO M. HAVU A. THORSTENSSON B SJÖDIN and J KARLSSON Skeletal muscle fibres and muscle enzyme activities in monozygous and dizygous twins of both sexes Acta physiol scand. 1977 100 385-392.

3 KOMI P V and J KARLSSON Skeletal muscle fibre types enzyme activities and physical performance in young males and females Acta physiol scand 1978 103 210-218

4 KARLSSON J P V KOMI and J.H T VIITASALO Muscle strength and muscle characteristics in monozygous and dizygous twins Acta physiol scand. 1979 In press

INTRODUCTION

Studies conducted on monozygous (MZ) and dizygous (DZ) twins have suggested that the genetic component is a primary determinant of the interindividual variation observed in maximal oxygen uptake and muscular power (Klissouras 1971; Komi et al 1973) Other recent reports (e.g Gollnick et al 1972) emphasize the interdependence between the subjects ability to perform work and their structural components such as skeletal muscle fibre composition

It seemed therefore logical to apply "the twin study concept as developed by Holzinger (1929) to i.e. human skeletal muscle especially to its histochemical and biochemical properties. The present study was undertaken to estimate in MZ and DZ twin pairs of both sexes the genetic component involved in selected physiological performances skeletal muscle fibre composition and enzyme activites In addition differences between sexes and interrelationships among the various parameters investigated were studied

Some of the data have been presented elsewhere separately (Komi et al 1976; Komi et al 1977; Komi et al 1978; Karlsson et al 1979) It was thought that it might be of interest to have all those data collected in one paper and in addition include the complete correlation matrices For this reason this Appendix has been prepared

METHODOLOGY

Subjects for the study were obtained through the Population Register of Finland This register provided the names and addresses of families with two or more children born on the same day in the city or surrounding communities of Jyväskylä between the years of 1950 and 1953 In addition one pair was obtained from Turku Finland and two pairs from Stockholm, Sweden. The final sample was composed of 20 male (9 MZ and 11 DZ) and 11 female (6 MZ and 5 DZ) twin pairs (Table 1) Initial determination of zygosity was performed by subjective

Table 1 Twin pairs distributed according to age and zygosity

Zygosity	Boys age (yrs)											Total pairs
	11	15	16	17	18	19	20	21	22	23	24	
MZ	1	2	1	2	-	-	2	-	1	-	-	9
DZ		2	2	1	-	3	-	2	-	-	1	11
Total pairs	1	4	3	3	-	3	2	2	1	-	1	20

Zygosity	Girls age (yrs)										Total pairs
	15	16	17	18	19	20	21	22	23	24	
MZ	1	1	-	1	-	-	-	1	1	1	6
DZ	-	-	1	1	1	1	1	-	-	-	5
Total pairs	1	1	1	2	1	1	1	1	1	1	11

observation of physical appearance Certainty of genetic identity or non-identity within the pairs was increased by serological analyses Venous samples of 7 to 8 ml of clotted blood was collected from each subject and further analysed in the Central Laboratory of the Red Cross Blood Transfusion Service (Helsinki) according to instructions of Race and Sanger (1969) and Giblett (1970) Antisera for the determination of the following red cell antigens were used:

A_1A_2BO MNS_s $Rh(CC^wDE_{ce})$ P_1 Lu^a K_k Fy^a and Fy^b and Jk^a

The serum was separated and frozen for later investigation

The following proteins and enzymes were determined

Haptoglobin Group specific substance Acid phosphatase (E C 3 1 3 2) myokinase (MK E.C 2 7 4 3) and Phosphoglucomutase (E C.2 7 5 1)

Those pairs of twins in which accordance in all the characteristics were found were considered monozygotic. In the 16 cases of observed dizygosity discordance was observed in more than five antigens or serum proteins.

Anthropometric measurements. In addition to recordings of body weight and height the following anthropometric measurements were performed: femorcondyle and radio-ulnar widths and skinfolds of subscapular, triceps brachii, biceps brachii, and suprailiac area (Durnin and Rahaman 1967). For correlative analysis fat free body weight was also estimated utilizing the method of von Döbeln (1959).

Maximal muscular power was determined using the method of Margaria et al (1966). In this measurement the subject ran maximally for a few seconds in a staircase. The running velocity (V) was measured electronically and converted to the vertical component (V_v). Mechanical power (kgm/sec) was computed on the basis of the subject's V_v, and his body weight. This test is referred to as an anaerobic test (Margaria et al 1966).

Muscular forces were measured as maximal voluntary isometric extensions of the right knee (quadriceps force) and of both legs (total leg force) respectively. The type of dynamometers and testing specifications emplyed have been reported elsewhere (Komi 1973; Komi and Viitasalo 1976). Force-time curve was registered during the total leg force measurements and the time to reach 70 % of the maximum force was taken as a force-time value.

Integrated electromyographic activity (IEMG) was picked up from both m rectus femoris and m. vastus lateralis during maximum right knee extension with Beckman miniature mixed surface electrodes and amplified with Brookdeal 9432 pre-amplifiers. After storage of the data on magnetic tape (Philips Analog 7 tape recorder) the processing of the EMG signals was performed with Hewlett-Packard 2116C computer system (Viitasalo and Komi 1975).

Chronaximetric measurements. Neuroton Model 626 stimulator was used to investigate the vastus lateralis muscle with constant current method using the following stimulus durations: 0.1 msec, 1 msec and 30 msec. The 30 msec duration was considered as a rheobase stimulus and was therefore used as a basis for calculation of the chronaxie value.

Aerobic power The standardized method described by Saltin and Astrand (1967) was used to determine the individual maximum oxygen uptakes ($\dot{V}O_2$max) during treadmill running Douglas bag technique was used for collection of expired air and CO_2 and O_2 contents were measured on a Schollander gas analyzer

Heart rate (HR) was recorded during the $\dot{V}O_2$max test and the HR-value at the termination of the run was taken as the peak heart rate

Peak blood lactate concentration was determined in arterialized finger tip blood samples taken 3 to 5 min after the maximal treadmill run. Reagents and instructions of Biochemica Boehringer GmbH was used for the determination of the whole blood lactate concentration.

Skeletal muscle fibre composition Two muscle biopsy samples were taken from the vastus lateralis muscle with a biopsy needle (Stille-Werner) as described by Bergström (1962) In this method the skin and underlying fascia were locally anesthesized and a small 5 mm wide cut was made so that the biopsy needle could be inserted into the muscle The site and depth of insertion were standardized for all subjects The first biopsy sample was used for classification of muscle fibres into fast twitch (FT) and slow twitch (ST) types (Gollnick et al 1972) by staining for ATPase as instructed by Padykula and Hermann (1955)

Muscle enzymes The second biopsy sample was used for determination of enzyme activities according to methods originally introduced by Lowry et al (1972) and modified as depicted in Table 2 Distribution of the heart muscle specific

Table 2   Summary table indicating specific conditions and literature references of the different enzyme activity analyses

Enzymes studied	Specific conditions	References
Ca^{2+} stim ATPase	In the presence of 2 mM Ca^{2+}	Thorstensson et al 1976 b
Mg^{2+} stim ATPase	In the presence of 2 mM Mg^{2+}	
CPK		Thorstensson et al. 1975
MK		and 1976 b
Phosphorylase (Plase)		Gollnick et al. 1975
$LDH_{py \rightarrow la}$		Karlsson et al 1968
$LDH_{la \rightarrow py}$		

LDH isozyme LDH-1 was determined in the muscle biopsy specimens as described by Karlsson et al (1974)

Statistical analysis In addition to the ordinary statistical procedures employed to calculate the means standard deviation (SD) and correlation coefficients (r) the single analysis of variance was used to test the significance of differences between the mean intrapair variances of the two twin types If the variance ratio (F) was significant at 5 % level of probability the computation of ther heritability estimate was done as originally described by Holzinger (1929):

$$Hest = \frac{S^2DZ - S^2MZ}{S^2DZ - S^2e} \qquad (1)$$

where S^2MZ and S^2DZ denote intrapair variability of an attribute in MZ and DZ twins respectively and S^2e signifies the variance due to experimental error Formulae given by Partanen et al (1966) were used for computation of S^2DZ and S^2MZ Hest gives an estimation of the relative contribution of the genetic component in explaining the variability in a given attribute

RESULTS

Appendix contains information from individual published articles (Table 3-6) supplemented with complete correlation matrices (Table 7-11) Tables 3-11 are presented after the reference list. Due to this approach sometimes very high correlations of obscure significance will be quoted in the text. They cannot be satisfactorily explained today but it is our hope that their presence will initiate further research and thus be explained in the future

Hereditary factors

Anthropometric measurements The male MZ twins in contrast to the females demonstrated smaller intrapair variations than their DZ counterparts (Fig 1)

Electromyographic and chronaximetric data Trends were present to smaller mean intrapair differences in male MZ twins as compared to male DZ twins (Fig 2) Similar trends were not present among females

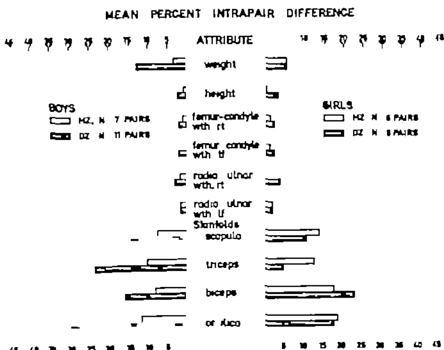


Fig 1 Mean percent intrapair differences in MZ and DZ twins for anthropometric data in boys (left) and girls (right)

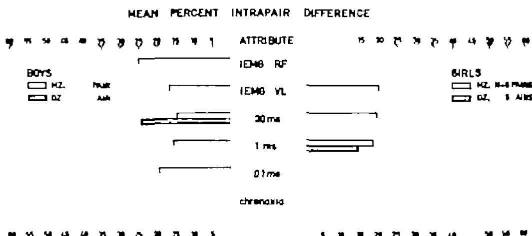


Fig 2 Mean percent intrapair differences in MZ and DZ twins for integrated electromyographic (IEMG) data from rectus femoris (RF) and vastus lateralis (VL) muscles; and chronaximetric data of different stimulus durations (30 ms 1 msec and 0 1 msec) from vastus lateralis muscle

Functional tests The intrapair difference for the various functional tests with the exception of muscle strength variables demonstrated smaller variations in male MZ twins as compared to DZ males (Fig 3)

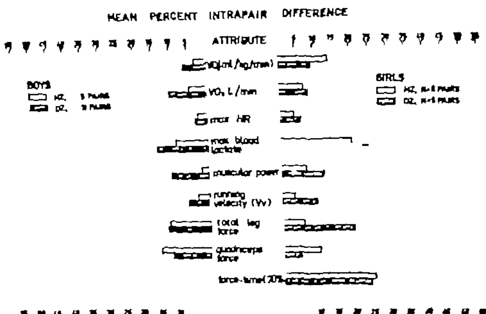


Fig 3 Mean percent intrapair differences in MZ and DZ twins for various performance data

The close correspondence of MZ male twins in performance variables were most pronounced for muscle power (Fig 4) and aerobic power expressed in $ml \times (kg \times min)^{-1}$ (Fig 5) In contrast to these variables measurements of e.g quadriceps force and total leg force demonstrated an almost equal scatter of points of the MZ and DZ pairs (Fig 6 and 7) The variance ratios (F-ratios) were however nonsignificant in all but one variable; muscular power in males and total sample and on a borderline significance in the case of VO_2max for males and total population.

Muscl fibre composition and enzyme activities The percent distribution of slow twitch fibres (ST %) in the vastus lateralis muscle was almost identical within MZ in contrast to DZ twin pairs (Fig 8 and 9) The Hest calculations (Table 4) for this attribute gave the following results: 99 5 % (males)

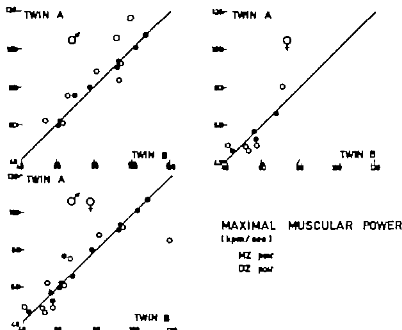


Fig 4 Intrapair comparison of maximal muscular power for MZ and DZ twins

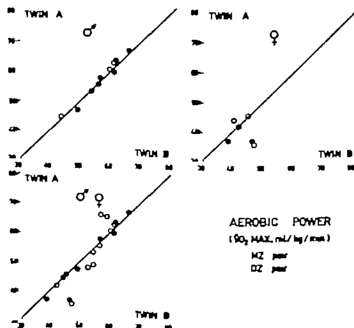


Fig 5 Intrapair comparison of maximal aerobic power expressed in ml x kg^{-1} x min^{-1} for MZ and DZ twins

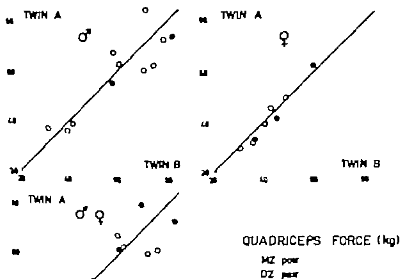


Fig 6 Intrapair comparison of quadriceps force for MZ and DZ twins

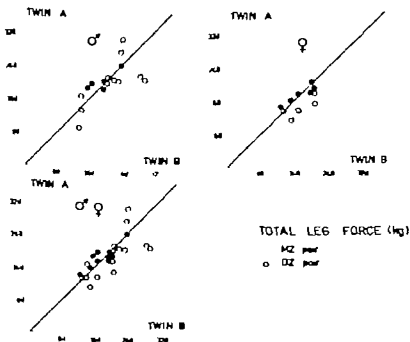


Fig 7 Intrapair comparison of total leg force for MZ and DZ twins

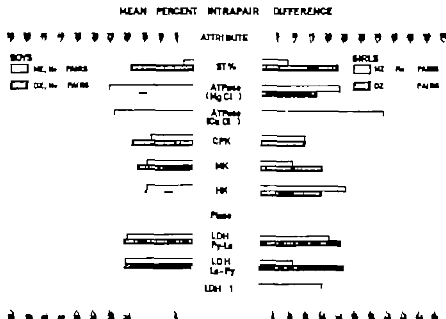


Fig 8 Mean percent intrapair difference in MZ and DZ twins for the distribution of slow twitch fibres and for activities of the different skeletal muscle enzymes

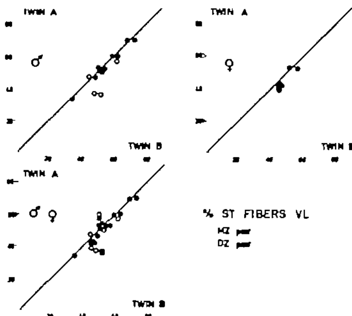


Fig 9 Intrapair comparison of slow twitch fiber distribution of m. vastus lateralis in MZ and DZ twins

92 2 % (females) and 96 5 % males + females indicating the presence of a genetic component The ST % values for the MZ pairs were within the error of measurements while those of the DZ pairs where randomly scattered. In contrast to the skeletal muscle fibre distribution none of the enzyme activities measured demonstrated any significant variability difference between MZ and DZ populations

Comparative analysis

For the purpose of comparative analysis correlations matrices were formed to obtain internal relationships between all the variables studied In the following only the most relevant information is taken from these matrices

Maximal oxygen uptake ($\dot{V}O_2max$) expressed in 1 x min^{-1} averaged 3 62 ± 66 and 2 23 ± 47 in the males and females respectively (Table 3) VO_2max was in the whole material disregarding sex differences best correlated to the following variables: muscular power (r = 90) quadriceps force (r = 75) running velocity (r = 70) total leg force (r = 62) and force time (r = = - 59) Similar correlations were obtained when the material was examined with regard to sex with the exception of running velocity (both sexes) and force time (males only)

When $\dot{V}O_2max$ was expressed per kg body weight (57 ± 6 and 43 ± 5 ml x (kg x min) ^{1} in males and females respectively) the best correlations were found to variables such as running velocity (males r = 57) and muscular power (females r = 52) When $\dot{V}O_2max$ was examined in relation to other variables with regard to zygosity it was found that $\dot{V}O_2max$ expressed in ml x (kg x min) ^{1} was correlated significantly to percent ST fibres (r = 50) in the MZ twins in contrast to the DZ twins

Muscular power was in the whole material as expected best related to different strength variables as well as force time (r = - 67 Fig 10) and in addition to VO_2max (r = 90 and 51 respectively for 1 x min^{-1} and ml x (kg x min) ^{1}) No significant relationship was however present between muscular power and percent distribution of ST fibres Similar relations were present in muscular power when the males and females were studied separately (the average values were 83 ± 18 and 57 + 10 kpm x sec^{-1} respectively) In addition muscular power in both sexes demonstrated a high correlation to percent fat (r = 66 and 73 in the females and males respectively)

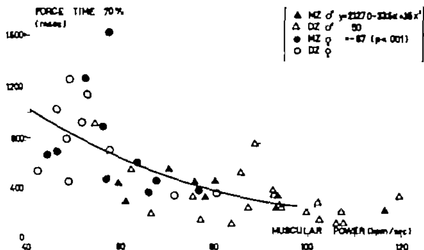


Fig 10 Relationship between value of force-time at 70 % of maximum isometric contraction (total leg force measurement) and maximal muscular power for all twin groups

The MZ twins (mean value 68 ± 16 kpm x sec^{-1}) demonstrated correlations between muscular power and VO_2max (r = 88 and 49 respectively for 1 x min^{-1} and ml x (kg x $min)^{-1}$) in contrast to the DZ twins (mean value 77 ± 22 kpm x sec^{-1}) The DZ twins were the only group however to demonstrate correlations to total leg force (r = 76) and IEMG of the vastus lateralis muscle (r = 46)

Quadriceps force was in the whole population best related to muscular power (r = 82) total leg force (r = 80) force time (r = - 46) and VO_2max (r = 75 and 36) respectively for 1 x min^{-1} and ml x (kg x $min)^{-1}$ Quadriceps force for the two sexes averaged 58 ± 16 and 40 ± 9 kp in males and females respectively and in terms of correlation matrix pronounced differences seemed to be present between them Thus in males total leg force (r = 82) muscular power (r = 82) VO_2max (1 x min^{-1} r = 69) % ST fibres (r = - 55 Fig 11) showed the highest correlations whereas Ca^{2+} stimulated ATPase (r = - 57) LDH-1 (r = 55) VO_2max (1 x min 1 r = 48) and muscular power (r = 47) were best correlated to quadriceps force in females

No differences in terms of correlations seemed to be present for quadriceps force when comparing monozygous (mean value 79 ± 4 kp) and dizygous twins (mean value 52 ± 16 kp)

VO_2max in ml x (kg x min)$^{-1}$ and muscular power (kpm x sec^{-1}) have both considered body dimensions because the weight is included in the calculations Quadriceps force on the other hand can also be related to body weight height squared skeletal weight *fat free body weight (FFW) or lean body mass (LBM)* If that is done significant correlations in addition to those presented above were obtained Thus in total population quadriceps force was related significantly to slow twitch fibre distribution (negatively) and running velocity (positively)

Differences between the sexes and the two twin materials were also present.

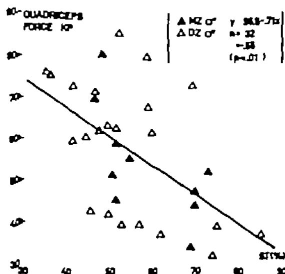


Fig 11 Relationship between quadriceps force and percent distribution of slow twitch fibers in vastus lateralis muscle of male MZ and DZ twins

Activities of enzymes involved in ATP turnover during muscle contraction The enzymes CPK MK and Ca^{2+} and Mg^{2+} stimulated ATP-ases demonstrated in the whole population only vague correlations to performance variables E g CPK was related to running velocity (r = 41) (Fig 12) and force time (r = - 40)

The two sexes differed in their correlation matrices Thus skeletal muscle MK activity in males in contrast to the females demonstrated significant

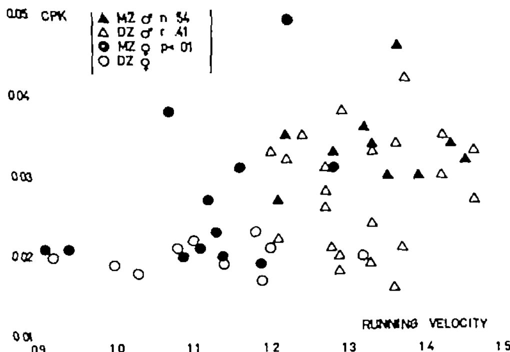


Fig 12 Relationship between the activity (in moles x g^{-1} x min^{-1} x 10^{-2}) of creatine phosphokinase (CPK) and "uphill running velocity (m/sec)

correlations to IEMG of rectus femoris (r = - 38) and muscular power (r = 35) On the other hand Ca^{2+} stimulated ATP-ase activity showed significant correlations only in the females (peak blood lactate; r = - 46 and quadriceps force r = - 57) as was also true for Mg^{2+} stimulated ATP-ase versus VO_2max (1 x min^{-1} r = 45) In absolute figures differences between the sexes were only present for Ca^{2+} stimulated ATP-ase (mean values 11 ± 10 and 06 ± 07 x 10^{-4} moles x g^{-1} x min^{-1} in males and females respectively) These enzyme activities and their relation to performance variables were not in general influenced by zygosity

Activities of enzymes involved in glucose residue metabolism. Hexokinase (HK) phosphorylase (Plase) and lactate dehydrogenase (LDH) showed no or very vague relationships to performance variables

In absolute figures a difference between sexes was present in Plase (males 1 05 ± 56; females 74 ± 43) and in LDH la → py (males: 70 ± 22; females: 54 ± 24)

Distribution of LDH enzyme patterns (% LDH-1) was in females related to quadriceps force (r = 55) and to VO_2max $1 \times min^{-1}$ (r = 45) When % LDH-1 was examined with regard to zygosity it showed correlation only with muscular power (r = - 62) in the MZ group In absolute terms the distributions of the isozyme LDH-1 were not significantly different between MZ and DZ twins but when the two sexes were compared differences were present (mean value 16.3 ± 12 2 and 25 3 ± 18 3 percent respectively for males and females)

Muscle fibre type distribution and enzyme activities In the whole material fibre type distribution was related significantly to the enzyme activities of MK (r = - 43) (Fig 13) and LDH (r = - 30) but not for ATP-ases

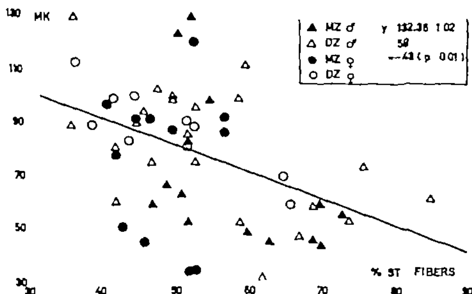


Fig 13 Relationship between the activity of myokinase (MK in moles $\times g^{-1} \times min^{-1} \times 10^{-6}$) and percent distribution of slow twitch fibres in vastus lateralis muscle for all subject groups

Correlation matrices demonstrated in general that the enzymes involved in ATP turnover during muscle contraction were positively correlated to glycolytic enzymes and negatively to aerobic metabolism as exemplified by % LDH-1 as well as the muscle s aerobic profile as expressed by its % ST distribution. The latter observation was pronounced in females where irrespective zygosity the percent distribution of LDH-1 increased with percentage of ST fibres (Fig 14) In males however no such relationships could be observed

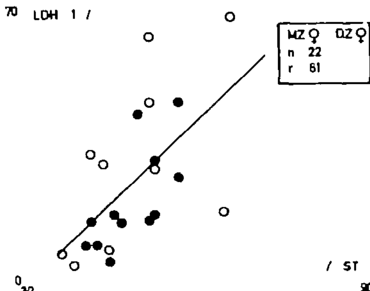


Fig 14 Relationship between LDH-1 isozyme (in % of total) and percent distribution of slow twitch fibres (% ST) in vastus lateralis muscle for female MZ and DZ

It must be pointed out however that the mean percent distribution of % LDH-1 was lower in the males (16 3 ± 12 2 %) than in the females (25 3 ± 18 3 %) in spite of a slightly higher percentage of ST fibres in the males (56 ± 12 %) than in the females (49 ± 8 %) Females also differed from males with a greater number of significant correlations of % LDH-1 to various enzymes

DISCUSSION

The most important finding in this project was the very strong heritability estimate observed for ST fibre distribution in all subjects In addition the results were able to confirm the earlier finding that the variability in muscular power among individuals is also genetically determined (Komi et al 1973) Furthermore the results demonstrated that muscular strength and IEMG did not show any strong genetic basis which is in accordance with Komi et al (1973)

Our results are however not in complete agreement with those of Klissouras (1971) who stated that the variability in aerobic power in males expressed as VO_2max (ml x (kg x min)$^{-1}$) is almost solely determined by heritability fac-

ture Computation of Best value assumes that the environmental influence are comparable among the twin pairs (Klissouras 1971) This was carefully taken into account in our previous study (Komi et al 1973) However no special control of the socio-economic health or physical activity status was made between the pairs or groups in the present study Although these environmental influences probably have been only minor they still might be the cause of the *finding* of no *genetic* component of e g the aerobic power measurements

The needle biopsy technique and the reproducibility of the results obtained in the specimens might be questioned Previous methodological studies on metabolites (Karlsson 1971) fibre distribution (Piehl 1974 and Thorstensson 1976) and enzyme activities (Gollnick et al. 1974) have demonstrated a good reproducibility in the investigated muscle (usually m. vastus lateralis) of the technique and methods applied in the present study

A question may be raised concerning the extent to which m. vastus lateralis is representative for the entire skeletal muscle pool and whether findings in that particular muscle can be generalized to other muscle groups Correlation coefficients for % ST fibre distribution between e g vastus lateralis and deltoideus muscles (r = in the order of 60 to 80) indicate that a relationship exists (Edström and Nyström, 1969 Saltin et al 1977 Rusko et al 1976)

From animal studies it is known that a motoneuron of a motor unit innervates a both histochemically (e g Bramlstatar and Lambert 1969) and physiologically (e.g Burke et al 1971) uniform type of muscle fibre There is to our understanding no reason to believe that the situation would be different in man. If that is the case it seems reasonable to suggest a similar or the same heritability control of the motor unit composition as for muscle fibre distribution. This means that by determining muscle fibre composition not only the metabolic profile but even the neuro-motoric control of the muscle is identified to a certain extent. Empirical evidence in man for this has already been published as relationship between VO_2max as well as type of sport activity and muscle fibre distribution in top athletes (Bergh et al 1978; Forsberg et al 1976; Rusko et al 1976) Moreover the significance of e g fast twitch (FT) fibres for muscle strength development has recently been documented experimentally in human skeletal muscle (Thorstensson t al 1976 a)

Studies where two muscles with different fibre population in the same individual have been examined have demonstrated that some enzyme activities (e g LDH PFK CPK and phosphorylase) are related to fibre type distribution (Gollnick et al 1974) In addition LDH isozyme pattern as well as MK activity have been demonstrated to be related to muscle fibres (Karlsson et al 1974 and Sjödin 1976) In the present study only MK and LDH activities and LDH isozyme pattern were in both or either sexes found to be related to muscle fibre type distribution The reason for this discrepancy is not clear By examining two different muscles in the same individual as was the case in Gollnick and his coworker s study it was possible to reduce the impact of individual differences in hormones nutritional status training etc. In the present study only one biopsy sample for each subject was obtained for enzyme analysis and for that reason individual differences in environment might play a greater role.

An example of environment is the individual training status As demonstrated by Thorstensson et al (1976 b) MK but not Mg^{2+} stimulated ATPase activity was related to muscle fibre population before strength training whereas after training also Mg^{2+} stimulated ATP-ase demonstrated a similar relationship One possible explanation could be that for the latter enzyme some genetic component related to fibre types was a prerequisite for the enzyme activity and changes due to training In this connection it is of interest to know whether the magnitude of training effects on the circulatory apparatus in one way or the other is related to muscle fibre population rather than activity level thus resulting in the high correlation reported for VO_2max and % ST fibres (Rusko et al 1976 Bergh et al 1978) in habitually physically active or homogenously trained individuals If that is the case it has to be suggested that also the type and magnitude of adaptive response might be influenced by a genetic predisposition as already suggested by Klissouras (1971)

Training has been reported to influence the value of VO_2max as much as 20-30 % (Saltin et al 1968 Ekblom 1969) Similar follow-up studies of anaerobic power have so far not been reported in the literature The high heritability for maximal muscular power (anaerobic power") suggests that it is less than aerobic power under the influence of environmental factors such as normal daily activity or training

The correlations between different performance variables were approximately the same in both sexes This has to be considered in spite of great differences in absolute values between the sexes Differences were however present between the sexes when muscle enzyme activities were correlated to performance variables. In addition to this significant correlations between the different enzyme activities were more frequent in females than in males

It has been demonstrated that a high percent of FT fibres is one prerequisite for performing a fast muscle contraction (Thorstensson et al 1976 a; Viitasalo and Komi 1978; Bosco and Komi 1979) In the present study it was demonstrated in the males that a relationship was present between muscular power and muscle fibre type distribution It is possible that one and the same biological phenomenon is the basis for the explanation of these findings. Support for this hypothesis is present in the observation of shorter force time with higher muscular power Force-time measurements as applied in the present study might be utilized in a similar fashion as dp/dt measurements in in vitro muscle studies I e. that approximately the same relationships are present in in situ studies in humans as have been demonstrated in animal studies

SUMMARY

The present study was designed to investigate with the twin study method the genetic influence on various traits of man physiological performance skeletal muscle fibre composition and enzyme activities 20 male pairs (9 monozygous twins (MZ) and 11 dizygous twins (DZ) and 11 female pairs (6 MZ and 5 DZ) ranging in age from 15 to 24 (one pair was 11 years) were used as subjects A total of 45 different parameters were investigated and interrelated.

The major findings and conclusions of the study were as follows:

(1) From the bulk of the physiological tests (muscle strength, muscular power electromyography chronaximetry aerobic power peak blood lactate peak heart rate) only the measurement of muscular power showed significant intrapair variance difference between male MZ and DZ twins The computed heritability estimate value (Hest) was 97 8 for this variable suggesting

Studies where two muscles with different fibre population in the same individual have been examined have demonstrated that some enzyme activities (e.g LDH PFK CPK and phosphorylase) are related to fibre type distribution (Gollnick et al 1974) In addition LDH isozyme pattern as well as MK activity have been demonstrated to be related to muscle fibres (Karlsson et al 1974 and Sjödin 1976) In the present study only MK and LDH activities and LDH isozyme pattern were in both or either sexes found to be related to muscle fibre type distribution The reason for this discrepancy is not clear By examining two different muscles in the same individual as was the case in Gollnick and his coworker s study it was possible to reduce the impact of individual differences in hormones nutritional status training etc In the present study only one biopsy sample for each subject was obtained for enzyme analysis and for that reason individual differences in environment might play a greater role.

An example of environment is the individual training status As demonstrated by Thorstensson et al (1976 b) MK but not Mg^{2+} stimulated ATPase activity was related to muscle fibre population before strength training whereas after training also Mg^{2+} stimulated ATP-ase demonstrated a similar relationship One possible explanation could be that for the latter enzyme some genetic component related to fibre types was a prerequisite for the enzyme activity and changes due to training In this connection it is of interest to know whether the magnitude of training effects on the circulatory apparatus in one way or the other is related to muscle fibre population rather than activity level thus resulting in the high correlation reported for VO_2max and % ST fibres (Rusko et al 1976 Bergh et al 1978) in habitually physically active or homogenously trained individuals If that is the case it has to be suggested that also the type and magnitude of adaptive response might be influenced by a genetic predisposition as already suggested by Klissouras (1971)

Training has been reported to influence the value of VO_2max as much as 20-30 % (Saltin et al 1968 Ekblom 1969) Similar follow-up studies of anaerobic power have so far not been reported in the literature. The high heritability for maximal muscular power (anaerobic power") suggests that it is less than aerobic power under the influence of environmental factors such as normal daily activity or training

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TABLE 3. MEANS ± SD OF THE DIFFERENT VARIABLES FOR THE *MONOZYGOUS*

	FEMALES		
	MZ	DZ	MZ+DZ
1. Anthropometric variables			
Weight (kg)	52.3 ± 8.8	49.1 ± 7.2	50.8 ± 7.7
Height (cm)	160.4 ± 6.6	163.2 ± 4.8	161.7 ± 5.9
Femur-condyle, lf	8.3 ± 0.3	8.1 ± 0.3	8.2 ± 0.3
widths (cm), rt	8.3 ± 0.3	8.1 ± 0.3	8.2 ± 0.3
Radio-ulnar lf	5.0 ± 0.2	4.9 ± 0.2	4.9 ± 0.2
widths (cm), rt	5.0 ± 0.2	4.9 ± 0.2	4.9 ± 0.2
Scapula skinfold (mm)	11.4 ± 4.2	8.1 ± 1.1	9.9 ± 3.6
Triceps skinfold (mm)	14.2 ± 4.2	12.0 ± 1.4	13.2 ± 3.5
Biceps skinfold (mm)	5.3 ± 2.4	4.9 ± 1.7	5.1 ± 2.1
Crista iliaca skinfold (mm)	7.4 ± 4.4	6.0 ± 1.6	6.7 ± 3.4
Fat (%)	23.6 ± 3.6	21.2 ± 2.1	22.5 ± 3.2
2. Performance variables			
$\dot{V}O_2$ max (l x min^{-1})	2.2 ± 0.4	2.2 ± 0.6	2.2 ± 0.5
$\dot{V}O_2$ max (ml x kg^{-1} x min^{-1})	41.8 ± 4.0	44.9 ± 5.2	43.2 ± 4.7
Peak heart rate	189.0 ± 8.9	191.3 ± 8.8	190.0 ± 8.7
Peak blood lactate	5.9 ± 2.0	7.1 ± 2.4	6.4 ± 2.2
Running velocity m x sec^{-1})	1.11 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Muscular power (kpm x sec^{-1})	58.0 ± 9.2	56.0 ± 11.9	56.6 ± 10.4
Total leg force (kp)	179.0 ± 28.8	182.2 ± 32.4	171.9 ± 30.0
Quadriceps force (kp)	40.8 ± 11.8	39.7 ± 7.3	40.3 ± 9.8
Force-time, (msec)	742.2 ± 371.8	755.1 ± 327.7	748.1 ± 344.2
3. Muscle fiber composition			
ST fibers (%)	48.4 ± 5.6	49.5 ± 10.1	49.1 ± 7.7
4. Muscle enzyme activities (moles x g^{-1} x min^{-1} x activity constant)			
Mg^{2+} ATP-ase 10^{-6}	9.4 ± 2.4	7.9 ± 2.7	8.7 ± 2.6
CPK 10^{-6}	0.09 ± 0.074	0.011 ± 0.026	0.06 ± 0.01
Ca^{2+} ATP-ase 10^{-3}	0.026 ± 0.009	0.020 ± 0.001	0.02 ± 0.01
MK 10^{-6}	75.1 ± 28.3	87.1 ± 15.6	80.6 ± 23.7
HK 10^{-6}	0.09 ± 0.03	0.11 ± 0.02	0.10 ± 0.02
[illegible]ase 10^{-6}	0.9 ± 0.5	0.5 ± 0.1	0.7 ± 0.4
[illegible] 10^{-4}	1.4 ± 0.3	1.6 ± 0.6	1.5 ± 0.6
LDH [illegible] 10^{-4}	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
LDH-1 isozyme (%)	20.6 ± 13.2	30.6 ± 22.7	25.3 ± 18.3
5. Electromyographic variables			
IEMG, rectus femoris (mV x sec^{-1})	380.9 ± 154.4	636.8 ± 222.8	498.1 ± 225.9
IEMG, vastus lateralis (mV x sec^{-1})	254.6 ± 81.6	313.6 ± 231.6	281.4 ± 165.5
6. Chronaximetric variables			
30 ms	4.0 ± 1.1	4.0 ± 1.07	4.0 ± 1.0
1 ms	5.2 ± 0.9	5.4 ± 1.09	5.3 ± 1.0
0.1 ms	16.4 ± 4.92	16.6 ± 5.7	16.5 ± 5.1

…Z) AND DIZYGOUS (DZ) TWINS OF BOTH SEXES

	MALES			
MZ	DZ	MZ+DZ	f	p<
66.3 ± 9.7	65.9 ± 12.7	62.6 ± 12.7	−3.99	.001
172.4 ± 6.7	177.5 ± 6.6	175.9 ± 6.9	−8.19	.001
9.4 ± 0.4	9.6 ± 0.4	9.5 ± 0.4	−12.17	.001
9.3 ± 0.5	9.5 ± 0.5	9.4 ± 0.5	−11.60	.001
6.6 ± 0.5	8.8 ± 0.3	5.7 ± 0.3	−9.88	.001
5.5 ± 0.5	5.8 ± 0.3	5.7 ± 0.4	−9.06	.001
6.7 ± 1.2	9.5 ± 5.1	8.6 ± 4.4	1.20	ns
4.9 ± 1.5	8.0 ± 3.5	7.0 ± 3.3	8.80	.001
3.3 ± 0.6	3.9 ± 2.3	3.7 ± 2.0	2.60	.01
4.8 ± 1.1	8.9 ± 5.8	7.6 ± 5.0	−0.84	ns
8.4 ± 2.3	12.5 ± 5.1	11.2 ± 4.6	9.85	.001
3.3 ± 0.5	3.8 ± 0.7	3.6 ± 0.7	−8.40	.001
55.9 ± 5.4	56.9 ± 5.9	56.6 ± 5.7	−9.42	.001
199.3 ± 12.5	193.1 ± 11.2	195.0 ± 11.6	−1.74	ns
7.9 ± 3.2	9.3 ± 2.3	8.8 ± 2.6	−3.60	.001
1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	−8.73	.001
73.8 ± 12.8	87.1 ± 18.3	82.9 ± 17.7	−6.63	.001
215.4 ± 52.7	213.7 ± 58.5	214.2 ± 54.5	−3.63	.001
53.8 ± 13.4	58.2 ± 16.6	56.8 ± 15.5	−4.67	.001
377.2 ± 91.7	376.0 ± 304.9	376.3 ± 255.7	5.41	.001
58.8 ± 10.3	54.6 ± 12.6	55.9 ± 11.9	−2.43	.05
10.7 ± 3.7	9.7 ± 3.4	10.0 ± 3.5	−1.40	ns
0.13 ± 0.062	0.028 ± 0.007	0.1 ± 0.1	−2.11	.05
0.03 ± 0.005	0.098 ± 0.119	0.03 ± 0.01	−3.17	.01
61.8 ± 17.1	79.9 ± 24.3	74.2 ± 23.6	0.34	ns
0.11 ± 0.03	0.09 ± 0.04	0.10 ± 0.04	−0.48	ns
1.0 ± 0.6	1.1 ± 0.5	1.1 ± 0.6	−2.21	.05
1.7 ± 0.8	1.3 ± 0.5	1.5 ± 0.6	0.34	ns
0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	−2.18	.05
14.2 ± 7.5	17.3 ± 13.8	16.3 ± 12.2	2.23	[illegible]
724.1 ± 215.6	555.4 ± 171.3	608.1 ± 199.2	−1.43	[illegible]
634.0 ± 672.4	504.0 ± 216.2	544.6 ± 408.3	−3.77	[illegible]
3.3 ± 1.0	4.1 ± 1.5	3.9 ± 1.4	−0.03	[illegible]
5.1 ± 2.0	5.7 ± 1.6	5.5 ± 1.8	−0.1	[illegible]
24.7 ± 23.1	16.5 ± 5.5	19.1 ± 13.8	0.3	[illegible]

Table 4. Estimates of variance within monozygous twins (S^2MZ), and dizygous twins (S^2DZ), variance of error of measurement (S^2e), and heritability of variation (Hest)

Attribute	S^2e	S^2MZ	S^2DZ	Hest
Maximal muscular power (kpm/sec)	1.20	3.31	95.05	97.8
Skeletal muscle fibre composition (% ST fibres)	2.15	2.24	94.62	99.5

Table 5. Summary of the correlation matrix for muscle biopsy variables showing the significant ($p < .05$) correlation coefficients for boys (left) and girls (right) including both monozygous and dizygous twins.

BOYS (MZ + DZ) below the diagonal; GIRLS (MZ + DZ) above the diagonal.

	% ST	ATPase Mg^{2+}	ATPase Ca^{2}	CPK	MK	HK	Pase	LDH p→l	LDH l→p	LDH_1
% ST									686	721
ATPase Mg^{2+}			475				569		608	
ATPase Ca^{2}				567			583		474	471
CPK						536	526		707	446
MK	509									
HK										
Pase									487	
LDH p→l			379			549				
LDH l→p					375					721
LDH								481	636	

Table 6. Summary of the correlation matrix for muscle biopsy variables showing the significant ($p < .05$) correlation coefficients for monozygous (left) and dizygous (right) twins of both sexes.

MZ Twins (♂ + ♀) — below diagonal; DZ Twins (♂ + ♀) — above diagonal

	% ST	ATPase Mg^{2+}	ATPase Ca^{2+}	CPK	MK	HK	Plase	LDH p→l	LDH l→p	LDH
% ST					488	374		.505		
ATPase Mg^{2+}				302						
ATPase Ca^{2+}							531			
CPK						395	531		561	444
MK								565		
HK										
Plase		519							.383	
LDH p→l						808			.358	621
LDH l→p	461		621	649						706
LDH			697						580	

APPENDIX

TABLE 7 CORRELATION MATRIX OF THE DIFFERENT

		1 21	2 22	3 23	4 24	5 25	6 26	7 27	8 28
Weight	1	1000							
Height	2	726	1000						
Femor-condyle, lf	3	711	826	1000					
Femor-condyle, rt.	4	748	836	963	1000				
Radio-ulnar lf	5	732	807	913	923	1000			
Radio-ulnar rt.	6	757	811	868	906	973	1000		
Scapula skinfold	7	649	— 27	82	74	68	124	1000	
Triceps skinfold	8	158	—412	—449	—419	—362	—333	724	1000
Biceps skinfold	9	147	—227	—213	—197	—201	—198	489	515
Crista ilica skinfold	10	665	145	259	295	301	328	889	560
Fat (%)	11	60	—508	—568	—531	—476	—436	678	941
$\dot{V}O_2$ max ($l \times min^{-1}$)	12	801	798	833	849	785	792	141	—269
$\dot{V}O_2$ max ($ml \times kg^{-1} \times min^{-1}$)	13	269	578	639	637	549	528	—359	—631
Peak heart rate	14	—211	77	21	— 18	—140	—152	—282	—290
Peak blood lactate	15	244	420	366	382	240	281	—103	—193
Running velocity	16	365	602	652	655	561	524	—163	—594
Muscular power	17	932	806	809	841	788	801	365	—114
Total leg force	18	725	601	667	700	601	621	356	— 29
Quadriceps force	19	807	652	702	740	663	698	291	— 96
Force-time, 70%	20	—489	—554	—682	—587	—551	—567	—132	207
ST fibers (%)	21	— 15	170	231	163	279	168	—246	—212
		1000							
ATPase Mg^{2+}	22	—144	— 77	118	97	— 74	— 41	— 61	—243
		— 55	1000						
ATPase Ca^{2+}	23	— 59	113	231	220	164	150	—126	—439
		— 72	117	1000					
CPK	24	24	171	293	270	262	231	—110	—361
		91	332	281	1000				
MK	25	204	12	— 67	— 34	— 46	25	265	367
		—396	— 27	—162	— 13	1000			
HK	26	124	106	57	62	— 96	— 39	12	— 68
		—198	163	24	—288	66	1000		
Pfase	27	—156	— 32	101	101	97	71	—122	—272
		75	375	368	458	— 33	—223	1000	
$LDH_{py \rightarrow la}$	28	— 96	— 66	— 95	— 69	—229	—206	— 37	— 86
		—342	233	212	—131	229	482	—164	1000
$LDH_{la \rightarrow py}$	29	192	222	229	233	201	161	121	—169
		—260	226	228	631	301	—132	307	296
LDH_1	30	— 23	— 83	—175	—160	— 97	— 76	— 4	279
		197	—228	—367	—426	— 19	95	—161	—416
IEMG, rf	31	— 86	32	137	69	122	108	—177	—248
		277	39	—103	— 10	—173	18	14	151
IEMG, vl	32	152	232	397	261	265	234	— 69	—261
		245	— 4	36	32	—124	— 19	— 75	—172
Chronaximetry/30 ms	33	206	174	— 35	18	52	43	198	215
		34	—244	— 78	—109	77	— 47	48	— 43
Chronaximetry/1 ms	34	43	79	9	— 28	39	11	— 32	28
		267	—245	—193	—122	—125	— 92	6	—106
Chronaximetry/0.1 ms	35	— 27	11	152	92	171	179	— 70	—100
		165	—109	— 80	229	23	—153	47	—130

VARIABLES FOR ALL SUBJECTS (n = 54)

Significance levels:

(x1000)	p<
450	.001
350	.01
270	.05

9 29	10 30	11 31	12 32	13 33	14 34	15 35	16	17	18	19	20
1000											
403	1000										
579	476	1000									
−93	321	−415	1000								
−335	−133	−755	731	1000							
−26	−229	−347	72	431	1000						
−20	93	−329	517	601	416	1000					
−175	30	−633	696	791	320	416	1000				
48	526	−201	903	507	−62	342	672	1000			
77	450	−89	620	260	−296	168	361	714	1000		
26	437	−163	750	360	−177	239	465	622	803	1000	
158	−265	264	−592	−434	−20	−361	−481	−565	−332	−463	1000
−248	−132	−338	96	262	266	67	149	26	−88	−146	−165
−52	−61	−237	8	211	300	59	170	−49	20	−51	−37
−229	−162	−355	140	274	141	−4	304	73	104	24	−63
−172	−66	−355	177	210	202	−45	414	175	−38	79	−396
333	336	326	60	−81	74	177	−143	106	−66	192	−35
−1	26	−3	57	−35	−45	36	−34	96	249	221	−66
−171	−66	−297	68	309	196	161	277	−32	−173	−116	−174
106	−48	−49	−109	−4	334	69	−20	−66	65	18	306
52	213	−161	223	167	332	166	362	292	45	153	−233
1000											
−33	0	237	−146	−148	−311	−124	−275	−132	−72	16	−30
−703	1000										
−220	−66	−300	−1	102	116	14	−7	−60	−2	−40	82
−174	−84	1000									
−69	17	−319	261	232	93	26	256	222	192	269	−227
17	−9	306	1000								
158	161	168	65	−61	7	157	−68	127	−42	−105	−206
254	−118	−224	−307	1000							
−31	26	−15	−11	−2	101	202	−6	23	−106	−161	−148
192	−165	54	240	620	1000						
18	−78	−133	18	36	92	46	50	−11	5	−62	−117
−2	10	250	278	19	278	1000					

APPENDIX

TABLE 9. CORRELATION MATRIX OF THE DIFFERENT VARIABLES

		1	2	3	4	5	6	7	8
		21	22	23	24	25	26	27	28
Weight	1	1000							
Height	2	757	1000						
Femor-condyle, lf.	3	772	858	1000					
Femor-condyle, rt.	4	791	841	893	1000				
Radio-ulnar lf.	5	739	838	948	944	1000			
Radio-ulnar rt.	6	783	837	930	933	964	1000		
Scapula skinfold	7	656	180	277	301	275	339	1000	
Triceps skinfold	8	166	—338	—387	—358	—356	—313	834	1000
Biceps skinfold	9	203	—157	—112	— 90	—158	—174	316	823
Crista ilica skinfold	10	691	189	378	406	404	454	938	540
Fat (%)	11	69	—463	—489	—450	—470	—423	566	938
$\dot{V}O_2$ max ($l \times min^{-1}$)	12	824	796	816	814	732	754	316	—234
$\dot{V}O_2$ max ($ml \times kg^{-1} \times min^{-1}$)	13	299	650	594	569	651	529	—250	—623
Peak heart rate	14	—221	— 2	—121	—170	—139	—164	—342	—238
Peak blood lactate	*15*	*310*	*436*	*358*	*349*	*217*	*280*	*24*	*—125*
Running velocity	16	536	610	680	678	681	636	66	—437
Muscular power	17	958	791	825	838	764	813	534	— 19
Total leg force	18	760	506	693	733	596	665	521	6
Quadriceps force	19	814	577	692	710	600	653	520	— 27
Force-time, 70%	20	—541	—493	—546	—556	—581	—584	—293	73
ST fibers (%)	21	37	296	201	175	336	236	—220	—162
		1000							
ATPase Mg^{2+}	22	— 15	— 18	222	189	109	171	34	—262
		—138	1000						
ATPase Ca^{2+}	23	63	199	349	349	348	350	— 9	—451
		— 1	66	1000					
CPK	24	210	189	371	339	447	383	106	—276
		144	362	212	1000				
MK	25	22	—220	—218	—225	—280	—199	176	304
		—488	— 9	— 20	— 70	1000			
HK	26	174	28	10	49	—105	— 4	128	141
		—374	118	— 98	—395	318	1000		
Pase	27	— 2	157	330	306	360	319	—110	—504
		— 29	278	631	531	—117	—207	1000	
$LDH_{py \rightarrow la}$	28	—177	—298	—198	—189	—277	—218	— 39	16
		—506	231	120	—202	585	262	—115	1000
$LDH_{la \rightarrow py}$	29	253	114	254	240	280	246	288	— 42
		—182	166	130	561	306	—213	383	356
LDH_1	30	—139	— 71	—237	—231	—199	—165	—124	192
		220	—195	—255	—444	—262	158	—178	—521
IEMG, rf	31	—247	—257	—185	—196	—162	—164	— 61	67
		—102	64	— 98	— 43	— 19	—171	—131	196
IEMG, vl	32	356	337	399	353	327	311	180	—163
		56	63	136	137	— 6	—117	95	—217
Chronaximetry/30 ms	33	112	163	19	24	79	35	18	106
		253	—396	— 36	— 48	— 69	— 29	31	—121
Chronaximetry/1 ms	34	83	143	14	13	98	63	— 35	74
		279	—412	—152	48	— 17	—132	— 48	— 62
Chronaximetry/0.1 ms	35	— 67	1	— 61	— 57	54	35	—136	26
		228	—202	—166	61	— 26	—115	—171	119

FOR ALL DIZYGOTS INCLUDING BOTH BOYS AND GIRLS (n = 32)

Significance levels

r (x1000)	p<
560	.001
450	.01
350	.05

9	10	11	12	13	14	15	16	17	18	19	20
29	30	31	32	33	34	35					
1000											
366	1000										
637	466	1000									
21	362	−342	1000								
−236	−166	−735	707	1000							
12	−381	−284	27	333	1000						
183	59	−289	520	441	280	1000					
−125	172	−606	764	761	56	324	1000				
113	569	−106	898	474	−161	341	748	1000			
221	565	− 3	616	289	−365	298	457	746	1000		
244	560	− 25	757	366	−248	261	624	853	833	1000	
224	−363	166	−637	−417	263	−264	−606	−566	−367	−434	1000
−165	−136	−249	67	209	318	− 70	127	67	−157	−229	−153
−116	27	−274	120	192	125	− 79	66	24	207	130	26
−339	− 18	−400	276	424	115	147	279	148	130	136	−144
−267	165	−344	377	347	79	−165	441	305	− 19	137	−404
372	140	316	− 20	−110	124	45	−141	− 27	−121	106	254
192	87	265	31	−261	−311	− 26	−141	115	303	262	− 3
−358	− 27	−616	236	442	80	19	327	89	− 45	69	−325
240	− 66	108	−202	−139	276	115	−190	−193	− 22	− 56	506
11	336	−132	277	209	330	137	325	298	79	173	−147
1000											
0	−139	219	−236	−166	−339	−245	−160	−168	−106	− 77	− 90
−788	1000										
− 57	− 56	111	−361	−367	4	−245	−422	−330	−191	−250	401
− 67	−168	1000									
164	203	−172	440	362	156	209	522	458	373	605	−233
176	− 21	−107	1000								
− 69	50	29	29	− 59	53	222	−133	33	−107	−228	−290
273	−158	−209	−125	1000							
− 55	47	4	− 45	−115	107	214	− 38	45	−102	−167	−196
					1000						
370	−246	−215	− 9	849	212	159	− 97	− 96	−104	−250	− 18
65	− 34	− 21	−199	−164	738	1000					
303	−305	− 69	−166	473							

APPENDIX

TABLE 10. CORRELATION MATRIX OF THE DIFFERENT VARIABLES FOR ALL

		1 21	2 22	3 23	4 24	5 25	6 26	7 27	8 28
Weight	1	1000							
Height	2	456	1000						
Femor-condyle, lf	3	765	388	1000					
Femor-condyle, rt.	4	798	434	952	1000				
Radio-ulnar lf	5	802	495	778	804	1000			
Radio-ulnar rt.	6	706	696	684	720	853	1000		
Scapula skinfold	7	568	−111	506	409	303	202	1000	
Triceps skinfold	8	794	26	628	587	446	367	803	1000
Biceps skinfold	9	313	− 69	109	100	42	− 24	573	317
Crista ilica skinfold	10	665	− 56	499	404	253	166	852	865
Fat (%)	11	714	− 60	556	504	360	235	910	905
$\dot{V}O_2$ max (l x min^{-1})	12	627	354	556	656	663	565	335	587
$\dot{V}O_2$ max (ml x kg x min^{-1})	13	331	284	234	304	433	331	−112	80
Peak heart rate	14	− 99	152	− 96	−162	−224	− 39	206	100
Peak blood lactate	15	231	396	131	256	35	312	−109	170
Running velocity	16	− 23	139	−126	−125	− 66	−185	62	−106
Muscular power	17	626	472	589	601	621	489	502	596
Total leg force	18	721	74	726	781	696	482	410	627
Quadriceps force	19	694	412	513	600	516	501	163	518
Force-time, 70%	20	−486	−421	−494	−674	−309	−446	−250	−300
ST fibers (%)	21	482	161	379	312	534	402	134	372
		1000							
ATPase Mg^{2+}	22	−326	−369	62	− 64	−305	−290	240	21
		−214	1000						
ATPase Ca^{2+}	23	−426	−374	−141	−256	−197	−317	− 13	−360
		−163	475	1000					
CPK	24	−124	145	126	60	−118	−145	10	−149
		−342	366	567	1000				
MK	25	164	207	248	254	− 62	17	220	312
		−103	313	−369	166	1000			
HK	26	− 11	− 60	− 82	− 26	− 74	105	− 82	24
		− 85	−113	−387	−636	124	1000		
Pfase	27	−110	−169	76	6	−196	−244	202	62
		−271	569	603	526	1	−424	1000	
$LDH_{py \to la}$	28	−280	−382	−313	−280	−242	−306	130	− 15
		−405	105	−139	−164	303	267	−166	1000
$LDH_{la \to py}$	29	−433	−117	−159	−146	−387	−367	− 6	−293
		−666	506	474	707	374	−277	467	404
LDH_1	30	476	244	196	205	317	325	− 16	315
		721	−266	−471	−448	20	312	−346	−416
IEMG, rf	31	−116	− 17	−261	−236	−116	− 51	−113	− 8
		106	−265	−522	−530	153	215	−334	416
IEMG, vl	32	30	53	−132	−206	−236	−161	− 51	139
		224	149	−326	−139	261	142	− 69	−169
Chronaximetry/30 ms	33	298	267	234	306	165	265	361	141
		−300	−115	−117	− 14	− 8	32	179	− 66
Chronaximetry/1 ms	34	− 20	−252	− 92	− 3	6	212	− 69	−143
		−464	−399	−174	−186	−169	239	73	142
Chronaximetry/0.1 ms	35	169	143	75	75	117	295	334	195
		−297	−202	−165	−126	62	193	27	352

GIRLS INCLUDING BOTH MONOZYGOTS AND DIZYGOTS (n=22)

9 29	10 30	11 31	12 32	13 33	14 34	15 35	16	17	18	19	20
1000											
504	1000										
624	931	1000									
324	503	580	1000								
48	79	46	658	1000							
66	308	142	−272	−45	1000						
22	186	125	411	267	201	1000					
433	39	113	224	420	207	72	1000				
519	572	656	828	520	11	236	541	1000			
113	437	646	725	229	−464	152	−144	522	1000		
−17	335	379	461	76	−144	274	−179	470	467	1000	
−476	−426	−445	−463	−102	−92	−562	−71	−462	−303	−435	1000
−145	230	212	394	388	−32	−259	−129	331	386	227	78
168	55	139	−449	−279	245	−298	69	−233	−235	−180	−9
141	−337	−189	−417	−212	−68	−402	252	−218	−230	−574	216
190	−110	−9	−340	−391	179	−52	261	43	−163	−78	−250
87	428	240	−90	−41	518	263	−90	74	−113	305	−337
−57	49	5	104	53	−179	386	−428	−236	−31	166	−278
242	22	168	−154	−186	162	−108	269	63	−48	−173	34
−81	92	2	−381	−299	236	−3	−186	−356	−180	−74	277
90	−118	−128	−540	−385	391	45	262	−225	−378	−230	−102
1000											
30	212	202	453	327	−134	11	−134	330	219	552	−193
−721	1000										
−232	−5	−128	−90	42	237	124	−338	−298	−45	−68	312
−273	66	1000									
−30	85	74	−78	−33	119	−113	−49	−14	−255	418	52
−281	604	192	1000								
500	189	282	307	−91	−194	131	−22	254	207	54	−353
−20	−170	138	−194	1000							
−43	−184	−153	137	−6	−81	489	−102	−70	67	−127	[illegible]
−2	−359	344	−380	621	1000						
268	234	243	128	−103	273	425	5	149	82	−35	[illegible]
80	−233	220	−384	419	868	1000					

Significance levels:

r (x1000)	p<
650	.001
540	.01
430	.05

APPENDIX

TABLE 11 CORRELATION MATRIX OF THE DIFFERENT VARIABLES FOR ALL

Variable	No.	1 21	2 22	3 23	4 24	5 25	6 26	7 27	8 28
Weight	1	1000							
Height	2	706	1000						
Femor-condyle, lf.	3	657	630	1000					
Femor-condyle, rt	4	724	664	938	1000				
Radio-ulnar lf.	5	643	586	735	769	1000			
Radio-ulnar rt.	6	705	548	705	767	947	1000		
Scapula skinfold	7	774	256	331	392	391	458	1000	
Triceps skinfold	8	724	284	205	277	394	433	874	1000
Biceps skinfold	9	412	101	193	214	159	158	413	486
Crista ilica	10	715	170	290	370	398	448	951	863
Fat (%)	11	835	309	345	422	483	534	938	936
$\dot{V}O_2$ max (l x min^{-1})	12	782	616	528	571	402	483	447	428
$\dot{V}O_2$ max (ml x kg^{-1} x min^{-1})	13	—360	—136	—245	—204	—393	—338	—499	—414
Peak heart rate	14	—455	—263	—443	—470	—640	—654	—442	—346
Peak blood lactate	15	— 11	53	—107	— 46	—266	—205	— 4	135
Running velocity	16	38	68	82	159	—184	—158	—163	—249
Muscular power	17	962	700	661	739	576	641	660	604
Total leg force	18	848	399	654	696	450	825	499	298
Quadriceps force	19	780	489	604	870	474	557	546	362
Force-time 70%	20	—262	—182	—174	—168	—254	—270	—284	—192
ST fibers (%)	21	—370	—193	—209	—281	— 50	—187	—332	—183
		1000							
ATPase Mg^{2+}	22	—263	—323	—176	—164	—429	—328	—124	—24
		— 97	1000						
ATPase Ca^{2+}	23	—166	— 60	12	32	— 86	— 68	—109	—34
		—171	— 75	1000					
CPK	24	—240	—401	—211	—206	— 68	—125	— 88	—14
		111	245	19	1000				
MK	25	378	140	22	101	164	275	299	42
		—509	—159	— 45	— 41	1000			
HK	26	182	242	177	206	—173	— 90	44	—15
		— 238	255	142	—224	74	1000		
Plase	27	—421	—457	—430	—362	—237	—249	—193	—20
		72	249	205	323	7	—171	1000	
$LDH_{py \to la}$	28	— 42	26	—111	— 54	—421	—329	—124	—19
		—343	305	379	—122	183	549	—153	100
$LDH_{la \to py}$	29	243	— 18	—147	—109	— 53	— 83	311	391
		—309	— 11	— 12	261	375	— 84	94	26
LDH_1	30	— 60	176	129	148	228	251	— 89	— 6
		89	—134	—245	—262	—145	— 22	109	—481
IEMG, rf	31	—314	—386	—119	—248	—157	—190	—163	—19
		263	125	— 27	214	—377	— 72	94	— 2
IEMG, vl	32	— 36	— 93	230	— 79	— 25	— 76	— 1	—10
		138	—138	— 33	—144	—183	— 47	—233	—19
Chronaximetry/30 ms	33	248	328	— 76	35	136	92	125	31
		166	—288	— 52	—150	116	— 73	15	— 2
Chronaximetry/1 ms	34	21	— 14	— 96	—191	— 27	—107	— 4	18
		448	—234	—233	—165	— 99	—163	— 38	—19
Chronaximetry/0.1 ms	35	—137	—169	118	— 20	136	127	—134	— 9
		236	—129	—112	316	38	—210	14	—25

[illegible] BOYS INCLUDING BOTH MONOZYGOTS AND DIZYGOTS (n=32)

Significance levels:

(x1000)	p<
560	.001
450	.01
350	.05

	9 29	10 30	11 31	12 32	13 33	14 34	15 35	16	17	18	19	20
[illegible]	1000											
[illegible]	466	1000										
[illegible]	530	936	1000									
[illegible]	265	342	497	1000								
[illegible]	−226	−506	−495	206	1000							
[illegible]	48	−452	−426	−126	611	1000						
[illegible]	229	5	0	285	563	431	1000					
[illegible]	−173	−148	−186	337	569	269	219	1000				
[illegible]	339	626	732	826	−187	−375	37	303	1000			
[illegible]	328	457	427	463	−227	−442	−66	174	668	1000		
[illegible]	390	487	633	668	−160	−425	−76	322	615	824	1000	
[illegible]	412	−206	−211	−286	22	277	147	−222	−312	−81	−161	1000
[illegible]	−176	−291	−271	−41	−48	319	−12	−157	−392	−406	−545	−47
[illegible]	−74	−131	−218	−158	240	274	81	−16	−251	−26	−191	156
[illegible]	−294	−158	−241	−32	202	132	−27	91	−137	37	−31	53
[illegible]	−231	−122	−106	−147	−37	101	−369	130	−193	−267	−206	−233
[illegible]	473	329	443	467	83	−97	264	−43	346	25	320	64
[illegible]	23	21	−13	69	−90	−3	−60	169	219	347	266	−44
[illegible]	−265	−171	−220	−274	287	136	110	−34	−414	−421	−374	−70
[illegible]	239	−107	−137	−62	167	396	126	128	−19	153	69	433
[illegible]	273	349	383	162	−3	221	2	60	231	−21	56	−19
[illegible]	1000											
[illegible]	−341	−92	−117	−104	−31	−390	−7	−31	−61	−29	7	−274
[illegible]	−638	1000										
[illegible]	−77	−179	−197	−443	−293	−35	−262	−297	−377	−165	−294	220
[illegible]	−302	−101	1000									
[illegible]	68	−42	−64	−27	−116	−12	−179	−43	−29	91	73	−80
[illegible]	−98	−66	264	1000								
[illegible]	−34	159	247	98	−23	97	224	−79	194	−117	−146	−233
[illegible]	463	−125	−434	−348	1000							
[illegible]	2	69	114	−151	−111	133	113	−94	−16	−165	−253	−211
[illegible]	262	−78	−77	318	636	1000						
[illegible]	3	−146	−125	−153	−91	33	−95	−90	−154	−67	−163	−61
[illegible]	−[illegible]	164	266	319	−50	223	1000					

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